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Specificity and Nature of Binding of Antimacrophage Serum

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Summary. Heterologous highly specific antimacrophage serum (AMS) was obtained by immunizing rabbits with macrophage membranes, and subsequently absorbing the antiserum on lymphocytes (in the presence of EDTA). This AMS was highly specific against macrophages as demonstrated by cytotoxicity assays and immunofluorescent staining.

Incubation of macrophages with AMS abrogated their phagocytic activity. This inhibition of phagocytic activity could be reversed by incubating AMS-treated macrophages either in normal serum or in trypsin.

The differences in distribution and location of antigenic determinants on the surfaces of macrophages and lymphocytes was demonstrated by fluorescent staining. Distinct surface antigens, absent on lymphocytes were detected on AMS-treated macrophages.

INTRODUCTION

Monospecific antimacrophage sera (AMS) may provide an important tool in elucidating the role of macrophages in the immune response. Several investigators reported that mice inoculated with AMS exhibited a suppressed immunological response to bacteriophage ϕX174 (Panijel and Cayeux, 1968), SRBC (Argyris and Plotkin, 1969) and stomatitis virus (Hirsch, Gary and Murphy, 1969). Likewise, Dyminski and Argyris (1969) reported that treatment with AMS prolonged allograft survival in mice. Other studies, however, did not demonstrate any change in the immune response in AMS-treated mice (Loewi, Temple, Nind and Axelrad, 1969; Despont and Cruchaud, 1969; Gallily, 1971). Results dealing with the specificity of AMS against macrophages were also controversial. While Panijel and Cayeux (1968) and Unanue (1969) claimed monospecificity of AMS against macrophages, Loewi *et al.* (1969) and Gallily (1971) showed that AMS cross-reacted with lymphocytes as well as with granulocytes. In the present investigation attempts were made to obtain an antimacrophage serum possessing both a high degree of specificity against macrophages and negligible cross-reactivity against lymphocytes. In addition, to elucidate the nature of the interaction of AMS with macrophages, phagocytic assays and immunofluorescent staining were employed. The results illustrate the reversible binding of AMS to macrophages and demonstrate differences in the location and concentration of antigenic determinants on the surfaces of macrophages and lymphocytes.

MATERIALS AND METHODS

Animals

BALB/c female mice, 3–5 months old, were used as donors of macrophages. Locally bred rabbits, 2–4 kg, were used as producers of antisera.

Isolation of macrophages

Macrophages were obtained from peritoneal exudates, 4 days after intraperitoneal (i.p.) injection of thioglycollate (Gallily and Feldman, 1967). The peritoneal cells were incubated in 120 mm diameter petri dishes (50×10^6 cells/dish) in Hanks's balanced salt solution (BSS) containing 10 per cent newborn calf serum (NBCS) (Microbiological Associates), at 37° in an atmosphere of 5 per cent CO_2 in air. The medium was changed after 24 hours. The cells were harvested 48 hours after seeding, by scraping them off gently with a rubber policeman. Differential counts showed that 98 per cent of the cells were macrophages. Viability of the cells was determined by staining with 0·4 per cent erythrosin B (Phillip and Andrews, 1959); 97 per cent of the removed cells were viable.

Preparation of macrophage membranes

Membranes were prepared from macrophages cultured *in vitro* for 48 hours, by the Tris method of Warren, Glick and Nass (1966).

Immunization

The 'regular' AMS was obtained by inoculating rabbits with macrophages which had been cultured for 48 hours *in vitro*. Each rabbit received a total of 1×10^9 macrophages, given in three weekly i.p. injections. The 'membranal' AMS was obtained by immunizing rabbits with macrophage membranes, each rabbit receiving membranes prepared from 1×10^9 cultured macrophages in Freund's complete adjuvant, in three weekly intradermal injections. The antithymocyte serum (ATS) was prepared by immunizing rabbits with two successive i.p. injections of 10^9 thymocytes, 14 days apart. All the rabbits were bled 1 week following the last injection. The sera were separated, inactivated (56° for 30 minutes) and stored at −20°.

Absorption

Membranal AMS was absorbed on lymph node lymphocytes at 4°, in the presence of ethylenediaminetetraacetate (EDTA) (Boyse, Hubbard, Stokert and Lamm, 1970); 1 ml AMS was absorbed on 5×10^6 lymphocytes. This antiserum is referred to as 'absorbed membranal' AMS.

Absorption and elution

1 ml of regular AMS was absorbed on $2{\cdot}5 \times 10^8$ peritoneal macrophages. The antiserum was recovered from the macrophages by elution with glycine-HCl buffer, pH 2·5 (Harris, Ogburn, Harris and Farber, 1963). This AMS is referred to as 'eluted' AMS.

Cytotoxicity Assays

Peritoneal macrophages ($2{\cdot}5 \times 10^6$ cells/plate) of BALB/c mice were cultured in Petri dishes (35×10 mm) in Hanks's BSS containing 10 per cent NBCS. After 24 hours, the medium was replaced by 10 per cent of various dilutions of AMS. The cells were incu-

bated for 1 hour, and 5 per cent (v/total volume) guinea-pig complement was then added. After additional incubation for 2 hours, the cells were stained with 0·4 per cent erythrosin B. The percentage of dead cells stained by the dye in a population of 200 counted cells was determined in quadruplicates. The cytotoxic titre was expressed as the dilution of AMS which caused the death of at least 50 per cent of the cells. The same procedure was followed in testing cytotoxicity of AMS against lymphocytes and granulocytes, with the exception that these assays were carried out in test tubes. Lymphocytes were obtained from BALB/c spleens. Granulocytes were collected from the peritoneal cavities of BALB/c mice, stimulated 14 hours earlier with thioglycollate.

Phagocytosis

Macrophages were cultured in Hanks's BSS containing 10 per cent NBCS. The medium was replaced after 24 hours with a medium containing 10 per cent AMS in Hanks's solution. The cells were incubated with the antisera for 1 hour, followed by the addition of either *Bacillus subtilis* (wild strain), or sheep red blood cell (SRBC) with or without anti-SRBC. The ratio of the bacilli or SRBC to macrophages was 20:1. After 1 hour of incubation, the macrophages were washed three times, fixed in methyl alcohol and stained with Giemsa. The percentage of cells which phagocytosized the bacilli or the erythrocytes was determined by counting 500–1000 cells in each sample.

Trypsin treatment following incubation with AMS

Peritoneal macrophages were grown *in vitro* for 24 hours in 10 per cent NBCS and Hanks's BSS. The medium was then replaced with 10 per cent AMS in Hanks's BSS. 1 hour later, the medium was removed and 0·02 per cent twice-crystallized trypsin solution (Sigma) in Hanks's BSS was added. The cells were incubated with trypsin for 6 minutes after which the trypsin was neutralized with NBCS. The cells were washed and *B. subtilis* in Hanks's BSS containing 10 per cent NBCS was added. The percentage of phagocytosis was determined after 1 hour of incubation.

Trypsin treatment prior to incubation with AMS

Peritoneal macrophages were grown *in vitro* in 10 per cent NBCS for 24 hours. The medium was removed and the cells were washed incubated for 6 minutes with 0.02 per cent trypsin in Hanks's BSS. After neutralizing the trypsin with NBCS, the cells were washed and incubated for 1 hour in a medium consisting of 10 per cent AMS in Hanks's BSS. One hour later *B. subtilis* was added and the phagocytic activity of the macrophages was determined.

Fluorescent staining

The indirect technique was used throughout. Fluorescent goat anti-rabbit globulin was prepared according to Sulitzeanu, Slavin, Karaman and Goldman (1967). The peritoneal macrophages, spleen lymphocytes and peritoneal granulocytes were incubated with AMS, washed and stained with fluorescent goat anti-rabbit globulin, according to the method described by Möller (1961).

RESULTS

CYTOTOXICITY AND SPECIFICITY OF VARIOUS AMS

The cytotoxicity of AMS prepared in various ways was compared. It was found (Table 1) that antisera obtained in rabbits injected with cultured macrophages (regular AMS)

showed a mean titre of 1:1000 against macrophages and 1:500 against lymphocytes and granulocytes. AMS prepared in rabbits by injecting macrophage membranes (membranal AMS) exhibited a high specific cytotoxicity against macrophages (titre of 1:200) and a low cross-reactivity against other cell types (1:10 for lymphocytes and 1:30 for granulocytes). AMS absorbed onto macrophages and then eluted by a glycine-HCl buffer (eluted AMS), showed a titre of 1:60 for macrophages and 1:10 for lymphocytes and granulocytes. Only the absorbed membranal AMS was specific against macrophages. In 1:100 dilution it killed more than 50 per cent of the macrophages, without demonstrable cytotoxicity against other cell types tested.

TABLE 1

CYTOXICITY OF VARIOUS ANTIMACROPHAGE SERA (AMS) AGAINST MACROPHAGES, LYMPHOCYTES AND GRANULOCYTES

Type of serum in the medium	Titre* of cytoxicity		
	Against macrophages	Against lymphocytes	Against granulocytes
Regular AMS	1:1000	1:500	1:500
Membranal AMS	1:200	1:10	1:30
Eluted AMS	1:60	1:10	1:10
Absorbed membranal AMS	1:100	Non-toxic	Non-toxic
NRS	Non-toxic	Non-toxic	Non-toxic

Average titres of three to five different sera.

* The dilution which killed at least 50 per cent of the cells.

TABLE 2

THE OPTIMAL DILUTION IN WHICH VARIOUS ANTIMACROPHAGE SERA (AMS) DEMONSTRATED HIGHEST CYTOTOXICITY AGAINST MACROPHAGES AND LOWEST CYTOTOXICITY AGAINST LYMPHOCYTES AND GRANULOCYTES

Type of serum in the medium	Optimal dilution	Percentage of dead cells*		
		Macrophages	Lymphocytes	Granulocytes
Regular AMS	1:1000	90	54	57
Membranal AMS	1:100	72	20	27
Eluted AMS	1:40	65	25	25
Absorbed membranal AMS	1:20	90	2	10
NRS	1:10	3	6	4

Mean of two to five sera.

* Per cent dead cells minus per cent of dead control cells (incubated in Hanks's BSS).

In Table 2, the percentages of dead cells resulting from treatment with serial dilutions of differently prepared AMS are presented. It can be seen that the regular AMS had the highest cytotoxicity against macrophages, though this was accompanied by the highest degree of cross-reactivity against lymphocytes and granulocytes. Eluted AMS had a low degree of cross-reactivity with other cell types, but its potency was greatly reduced. Membranal AMS demonstrated a high cytotoxicity against macrophages with a low cytotoxicity against lymphocytes (a 1:100 dilution killed 72 per cent of the macrophages and 20 per cent of the lymphocytes). The absorbed membranal AMS which killed 90 per cent of the macrophages in 1:20 dilution was not cytotoxic at all to lymphocytes and granulocytes. This AMS was specific against macrophages.

EFFECT OF AMS ON PHAGOCYTOSIS

AMS abolished the ability of macrophages growing in culture to phagocytosize *B. subtilis* and SRBC, as shown in Table 3. 44 per cent of the macrophages growing in 10 per cent NBCS phagocytosized the bacilli after 1 hour of incubation. This phagocytic ability was markedly reduced (to 2–5 per cent) when the macrophages were incubated for 1 hour with 10 per cent AMS, before the addition of the bacilli. Antithymocyte serum (ATS) caused a partial inhibition, lowering the phagocytic rate to 20 per cent.

TABLE 3

EFFECT OF ANTIMACROPHAGE SERUM (AMS), ANTITHYMOCYTE SERUM (ATS) AND NORMAL SERA ON THE PHAGOCYTIC ACTIVITY OF MACROPHAGES *in vitro*

Incubation of macrophages	Percentage of phagocytosis		
	Bacillus subtilis	SRBC	SRBC with anti-SRBC
AMS 1*	3	3	2
AMS 2*	5	2	1
AMS 5*	2	Not tested	Not tested
ATS	20	3	6
NRS	42	87	94
NBCS	44	10	80

* Regular AMS.

AMS similarly inhibited the phagocytosis of SRBC by macrophages when conditions identical to those described above were employed. Only 2–3 per cent of the AMS-treated macrophages engulfed SRBC, as compared to 87 per cent of the macrophages grown in NRS. The addition of anti-SRBC did not prevent the inhibition of phagocytosis which was caused by AMS or ATS treatment. ATS affected phagocytosis in a similar way, abrogating almost completely the phagocytosis of SRBC. Apparently, the phagocytosis of SRBC by macrophages is more sensitive to the inhibitory effect of ATS than the phagocytosis of *B. subtilis*.

RECOVERY FROM PHAGOCYTIC INHIBITION

Cultured macrophages which had been incubated for 1 hour with AMS regained their ability to phagocytose after repeated washings and replacing of the medium with Hanks's BSS containing 10 per cent NBCS. At various time intervals after replacing the AMS with NBCS, *B. subtilis* was added, and the percentage of cells that engulfed the bacilli was scored. Fig. 1 demonstrates the kinetics of the recovery from the AMS effect. It can be seen that 2 hours after the removal of AMS the phagocytic rate began to increase, and within 24 hours the macrophages regained their regular phagocytic activity (about 50 per cent). Likewise, macrophages previously treated with ATS regained their normal ability to phagocytosize when transferred to medium containing normal serum. This recovery, however, appeared much earlier than that observed in the AMS-treated macrophages. The phagocytic activity of the macrophages reached its normal value 2–4 hours after replacing the ATS.

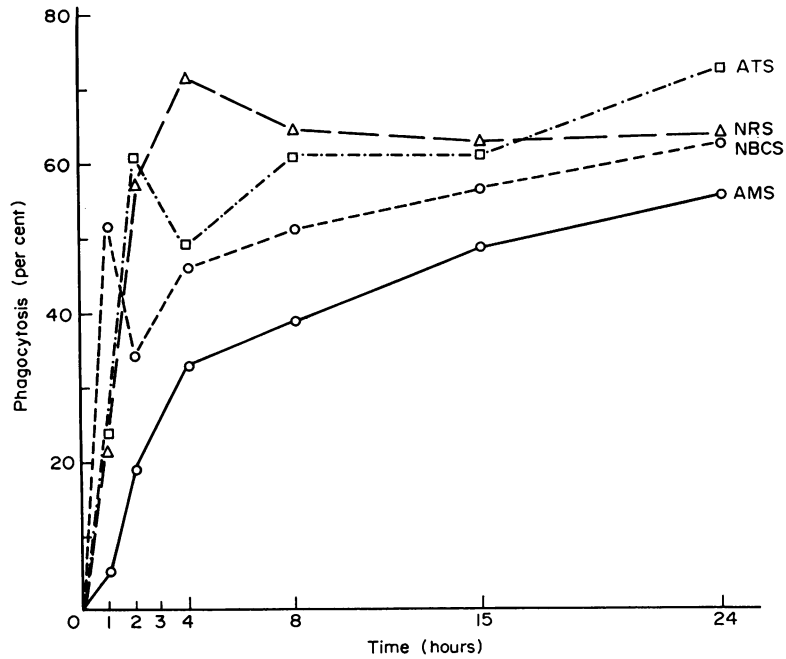

FIG. 1. Recovery of the phagocytic activity of macrophages treated with AMS or ATS. Macrophages were incubated with the different antisera or normal sera prior to incubation with medium containing new born calf serum. At various times thereafter, the percentage of macrophages which phagocytosized *Bacillus subtilis* was scored.

TABLE 4

THE EFFECT OF TRYPSIN ON THE PHAGOCYTOSIS OF *Bacillus subtilis* BY MACROPHAGES PREVIOUSLY TREATED WITH ANTI-MACROPHAGE SERA

Incubation of macrophages*	Percentage of phagocytosis	
	Without trypsin treatment	After trypsin treatment
Regular AMS	5	47
Membranal AMS	8	46
NRS	50	45
NBCS	52	46
Hanks's BSS	51	46

* Macrophages incubated with AMS or normal sera and then treated with 0·02 per cent trypsin for 6 minutes. The phagocytosis was scored after incubating the cells in medium containing 10 per cent NBCS.

TRYPSIN TREATMENT

Cultured macrophages incubated with regular AMS or membranal AMS lost almost completely their ability to phagocytosize *B. subtilis* (Tables 3–5). However, when AMS treated macrophages were incubated for 6 minutes with trypsin, the cells regained their ability to phagocytosize the bacilli. It can be seen in Table 4 that while only 5–8 per cent of AMS-treated macrophages phagocytosize the bacilli, 45–47 per cent of the cells showed

phagocytosis after trypsin treatment. The trypsin treatment did not affect the phagocytic activity of macrophages incubated in normal sera. In another experiment, in order to prevent the blocking of phagocytosis by AMS, trypsin was added to the macrophages before the AMS treatment. The increase in phagocytic activity of macrophages is illustrated in Table 5. 17–19 per cent of the macrophages treated with trypsin prior to AMS exhibited phagoctyosis, as compared with 5–7 per cent phagocytosis in cells treated with AMS only. However, this increase of phagocytic activity of trypsin-AMS-treated macrophages did not reach the values of the macrophages incubated in normal serum. The effect of trypsin on the macrophages could be either to cleave a portion of the surface antigenic determinants, or to reveal new determinants. In either instance the AMS would not recognize these changed or new surface antigens, and its binding efficiency to the macrophages would be reduced.

TABLE 5

THE PHAGOCYTIC ACTIVITY OF MACROPHAGES TREATED WITH TRYPSIN PRIOR TO THE ADDITION OF AMS

Incubation of macrophages	Percentage of phagocytosis	
	Without trypsin treatment	Trypsin treatment*
Regular AMS	5	17
Membranal AMS	7	19
NRS	41	35
NBCS	42	37
Hanks's BSS	43	42

* Macrophages treated with 0·02 per cent trypsin before incubation with AMS.

INDIRECT IMMUNOFLUORESCENT STAINING

Macrophages, lymphocytes and granulocytes were incubated with various preparations of AMS, and then stained with fluorescent goat anti-rabbit globulin. The dilutions of AMS giving the brightest fluorescent staining were 1:4 and 1:10. No difference in fluorescent staining was demonstrated with regular or membranal AMS. However, variations in staining of different cell types could be distinguished. Generally, the macrophages showed a typical strong granular staining all over the surface of the macrophage (Fig. 2). On the other hand, lymphocytes were stained quite differently; a thin ring-like fluorescent staining was distinguishable around the periphery of the cells (Fig. 3). Some lymphocytes showed fluorescent caps located on one pole of the cell, and in few cases dotting was observed instead of capping. Granulocytes were stained very similarly to lymphocytes. At no time was granulation demonstrated on lymphocytes or granulocytes. The absorbed membranal AMS stained only the macrophages, though the staining was much lighter than that obtained with macrophages incubated with membranal AMS. The absorbed membranal AMS did not stain the lymphocytes or the granulocytes at all, thus proving its high specificity against macrophages. In addition, it was found after trypsin incubation that AMS-treated macrophages were not stained by the fluorescent anti-rabbit serum. This indicates that AMS is no longer present on the macrophage membranes after trypsin treatment.

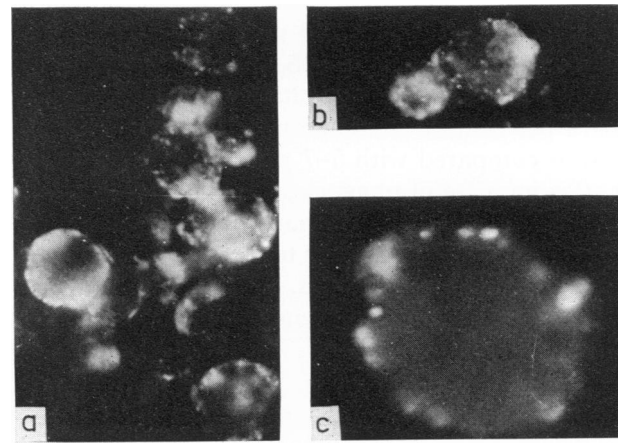

FIG. 2. Photomicrographs of indirect immunofluorescence of BALB/c macrophages. Living macrophages treated with AMS followed by fluorescein conjugated goat anti-rabbit globulin show a typical granular fluorescent staining all over their surface (a, b, × 680; c, × 1700). The controls treated with normal rabbit serum did not exhibit any staining.

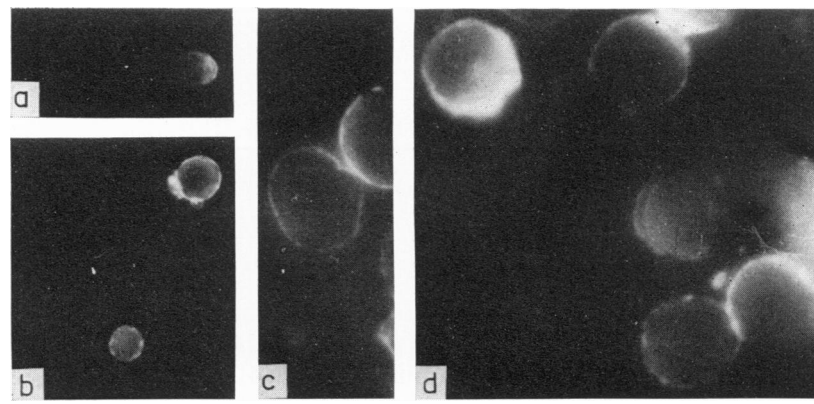

FIG. 3. Photomicrographs of indirect immunofluorescence of BALB/c spleen lymphocytes. The living lymphocytes were treated as described in Fig. 2. Typical fluorescent ring or fluorescent caps are illustrated (a, b, × 680; c, d, × 1700). The controls were treated with normal rabbit serum and did not exhibit any staining.

DISCUSSION

An antimacrophage serum highly specific against macrophages was obtained by injecting rabbits with macrophage membranes and subsequently absorbing the antiserum on lymphocytes in the presence of EDTA. The absorbed membranal AMS showed high cytotoxicity against macrophages with no detectable cytotoxicity against lymphocytes or granulocytes. Absorption of AMS on lymphocytes at room temperature in the absence of EDTA lowered the titre of cytotoxicity, but did not affect grossly the cross-reactivity of AMS against lymphocytes. 1 ml of regular AMS absorbed onto 5×10^8 lymph node lymphocytes (without the addition of EDTA) exhibited cytotoxicity in 1 : 100 dilution to 68 per cent of the macrophages and 53 per cent of the lymphocytes. It is apparent that the

presence of EDTA in the absorption mixture permitted successful binding of cross-reacting antibodies to lymphocytes, thus leaving an antimacrophage serum with a high degree of specificity. The exact mechanism by which EDTA exerts this effect is unknown; however one of the known functions of EDTA is to eliminate free divalent cations. It is suggested that during the absorption of AMS on lymphocytes the divalent cations caused the release of unidentified cytotoxic factors from lymphocytes into the antiserum. These factors might be responsible for the undiminished cytotoxicity which was observed against lymphocytes and macrophages. The elimination of these cations by EDTA prevents leakage of these agents, thus reducing the nonspecific lysis of the cells during the cytotoxic assay.

The failure of AMS to suppress *in vivo* the immune response reported by several independent studies (Loewi *et al.*, 1969; Despont and Cruchaud, 1969; Gallily, 1971) could be due to the reversibility of AMS binding to macrophages demonstrated in the present study. It is tempting to assume that in AMS-treated animals inhibition of engulfing and attachment of antigens to macrophages is only temporary and that recovery of these functions is followed by a normal immune response.

The most likely interpretation of our fluorescent staining experiments employing unabsorbed AMS is that localization and concentration of antigenic determinants on the membranes of macrophages differ from those of lymphocytes or granulocytes. Furthermore, the absorbed membranal AMS revealed preferentially antigenic determinants specific for macrophages. This specific AMS failed to detect antigenic determinants on lymphocytes or granulocytes. It is therefore suggested that this highly specific AMS binds to antigenic determinants located only on the macrophage membranes.

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