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BASIS OF TETRACAINE-INDUCED INHIBITION OF PHAGOCYTOSIS BY CULTURED MOUSE PERITONEAL MACROPHAGES

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Abstract—The inhibition of mouse peritoneal macrophage phagocytosis by the tertiary amine anesthetic, tetracaine, was studied *in vitro*. By investigating separately the recognition and ingestion phases of phagocytosis using ^{51}Cr-labeled sheep red blood cells (SRBC), the following observations were made. First, immune-mediated recognition of the SRBC via the opsonizing mouse immunoglobulin by the macrophages is inhibited by tetracaine. Second, non-immune recognition of unopsonized SRBC is not inhibited by tetracaine. Third, ingestion of the opsonized SRBC is inhibited by tetracaine to a greater extent than unopsonized SRBC. Fourth, the basis for the inhibition of recognition appears to be steric, probably related to anesthetic-induced clustering of receptors on the macrophage surface. Fifth, the results are discussed in terms of transmembrane control of macrophage receptors.

We have demonstrated recently an inhibition of phagocytosis of opsonized sheep red blood cells by cultured mouse peritoneal macrophages with the tertiary amine local anesthetics, tetracaine, dibucaine and procaine [1]. The ability of tertiary amine local anesthetics to disrupt the cytoskeletal network of cultured cells [2–4], coupled with the participation of microtubules and microfilaments in phagocytosing macrophages [5], implied that the observed inhibition of phagocytosis was the result of disruption of the macrophage cytoskeletal system.

The process of macrophage phagocytosis of particulate matter can be divided arbitrarily into three events: recognition of the particle, ingestion, and digestion. Our initial study did not distinguish on which event of phagocytosis tertiary amines exerted their inhibition [1]. However, since the above experiments employing opsonized SRBC were of short duration, the observed inhibition could be narrowed down to interference with either opsonin recognition or ingestion or both. Therefore, we sought to determine the event at which local anesthetic-induced inhibition occurred and the basis of this inhibition.

MATERIALS AND METHODS

Reagents. Tetracaine HCl was purchased from K & K Laboratories, Inc. (Plainview, NY), in crystalline form, and dissolved in CMEM (see below) immediately prior to use. The pH of the solution was adjusted to 7.2 by the addition of $NaHCO_3$. $Na_2[^{51}Cr]O_4$ (sp. act. 380 mCi/mg) was purchased from New England Nuclear (Boston, MA). Carrier free $Na[^{125}I]$ was purchased at 20 mCi/ml from the Amersham Corp. (Arlington Heights, IL).

Animals. Inbred C57BL/6 mice were used throughout this study as macrophage donors.

Preparation and purification of macrophage cultures. Macrophages were obtained from normal C57BL/6 mice. Three ml of thioglycollate medium (Baltimore Biological, Cockeysville, MD) was injected intraperitoneally into each mouse, and 5 days later the animals were killed by ether inhalation. Their peritoneal exudate cells (PEC) were harvested by lavage with Hanks' balanced salt solution (HBSS) (Grand Island Biological, Grand Island, NY). The PEC were centrifuged at 250 *g* and resuspended in Eagle's minimum essential medium supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, penicillin–streptomycin, L-glutamine and 2X the vitamins, designated CMEM (Flow Laboratories, Rockville, MD). PEC were plated into 16-mm diameter polystyrene culture dishes (Costar, Cambridge, MA) at a concentration of 5×10^5 PEC/dish in 1.0 ml. After a 30-min incubation at 37° in an atmosphere of 5% CO_2, the resultant monolayers were rinsed to remove nonadherent cells and refed with an equal volume of CMEM. More than 95 per cent of the remaining adherent cells were capable of phagocytosing carbon, exhibited typical macrophage morphology, and were used in the subsequent studies [6].

Preparation of opsonized and unopsonized ^{51}Cr-labeled SRBC. SRBC were collected in Alsever's solution (Grand Island Biological), stored at 4°, and were used 3–6 weeks later. Opsonization was accomplished by a 60-min incubation at 37° of 50 ml of a 2% (by volume) SRBC suspension with 0.1 ml of a 1:10 dilution of mouse anti-SRBC serum. The antiserum was prepared by injecting mice i.p. 7 days earlier with 0.25 ml of a 10% (by volume) washed SRBC suspension. A similar incubation for the preparation of unopsonized SRBC was carried out using normal mouse serum. The suspensions were centrifuged, and the supernatant fractions discarded. Radioactive labeling of the SRBC was accomplished by adding 200 μCi $Na_2[^{51}Cr]O_4$ (1 mCi/ml) for an incubation period of 2 hr at 37°. The SRBC were then washed three times in 50 ml of cold (4°) HBSS to remove cell free ^{51}Cr. At this time the supernatant fraction had no ^{51}Cr-activity. The final volume was adjusted to yield a 2% (by

volume) suspension of opsonized or unopsonized SRBC.

Quantitative assay for macrophage recognition of SRBC. Recognition was quantitated by the method of Rabinovitch [7] which distinguishes between the attachment and ingestion phases of phagocytosis. Twenty-four hr after the initial plating, the macrophage cultures were rinsed and the CMEM was replaced by 1.0 ml CMEM containing various concentrations of tetracaine. After a 2-hr incubation, the macrophage cultures were rinsed and refed with 1.0 ml CMEM. Two-tenths ml of 2% opsonized or unopsonized ^{51}Cr-labeled SRBC was added to each macrophage culture. The plates were swirled to ensure uniform distribution of the SRBC. The surface of the culture dish was covered completely with SRBC following the addition of 0.2 ml of a 2% suspension. After a 20-min incubation at 20°, the cultures were washed three times with 1.0 ml HBSS to remove unattached SRBC. The remaining adherent cells were lysed by incubating the monolayer with 1.0 ml of 1.0 N NaOH at 37° for 1 hr. The lysate was pipetted directly into 16 × 125-mm polystyrene test tubes (Falcon Plastics, Oxnard, CA), and radioactivity was determined using a Searle Analytic model 1185 counter with a NaI (T1) well-type detector.

Quantitative assay for macrophage ingestion of SRBC. Twenty-four hr after the initial plating, the macrophage cultures were rinsed and refed with 1.0 ml CMEM containing various concentrations of tetracaine. After a 2-hr incubation at 37°, macrophage cultures were rinsed and refed with 1.0 ml CMEM. Two-tenths ml of 2% opsonized or unopsonized ^{51}Cr-labeled SRBC was added to each macrophage monolayer and incubated for 2 hr at 37°. Radioactivity at this time was determined as above.

Preparation of heat aggregated [^{125}I]IgG. Purified mouse IgG was a generous gift of Dr. Gregory Warr. Ten μl of a stock IgG solution containing 3.7 mg/ml was labeled with carrier free (10 mCi/ml) Na[^{125}I] by the chloramine T method as described previously [8]. The iodinated protein was separated from free iodine by gel filtration on Bio Gel P2 (Biorad Labs, Richmond, CA). The absence of low molecular weight radioactivity was verified by precipitation with 10% trichloroacetic acid. One hundred μl of labeled material was added to 1.0 ml of the stock solution of unlabeled IgG and heat aggregated for 30 min at 60° after the method of Preud'homme and Seligmann [9].

Recognition of heat aggregated [^{125}I]IgG. Peritoneal efudate cells (8×10^4) were plated into Microtest II wells (Falcon, Oxnard, CA) and refed 30 min later with 0.1 ml CMEM. Twenty-four hr after the initial plating, the macrophage cultures were rinsed and refed with either 0.1 ml CMEM or 0.1 ml CMEM containing 100 μM tetracaine. After a 2-hr incubation at 37°, the macrophage cultures were rinsed and 0.05 ml of the heat aggregated [^{125}I]IgG, prepared as described above, was added to each culture. After a 20-min incubation at 20°, the cultures were washed three times with 0.1 ml HBSS to remove unattached IgG.

Calculation of percentage inhibition. Per cent inhibition =

$$1 - \frac{\text{cpm } ^{51}\text{Cr test macrophage}}{\text{cpm } ^{51}\text{Cr normal macrophage}} \quad 100$$

Statistical analysis. Results were analyzed for statistical significance by Student's *t*-test (two-tailed).

RESULTS

The present studies used tetracaine alone since the inhibition of phagocytosis of opsonized SRBC was probably via the same mechanism for tetracaine, dibucaine and procaine [1].

Inhibition of opsonin recognition. The recognition of opsonized SRBC for phagocytosis by macrophages occurs, presumably, by attachment of the Fc region of the opsonizing antibody to the macrophage surface (reviewed in Refs. 10 and 11). The first experiment, therefore, compares the recognition, as defined by Rabinovitch [7] of opsonized vs unopsonized SRBC for a 20-min incubation at 20°. It may be seen (Table 1) that, as expected, untreated macrophages recognize, to a greater extent, opsonized rather than unopsonized SRBC. Pretreatment of the macrophages for 2 hr with

Table 1. Inhibition of macrophage opsonin recognition of ^{51}Cr-labeled SRBC by tetracaine

Treatment concentration	SRBC condition	Average ^{51}Cr-activity in lysate at 20 min* (cpm ± S.D.)
None, CMEM	Opsonized	1346 ± 247
Tetracaine (100 μM)	Opsonized	637 ± 89† (53)‡
None, CMEM	Unopsonized	431 ± 139
Tetracaine (100 μM)	Unopsonized	632 ± 211§

* Macrophage cultures were incubated for 2 hr in 100 μM tetracaine or CMEM after which the attachment assay was incubated in CMEM; 0.2 ml of a 2% opsonized or unopsonized SRBC suspension, labeled with 200 μCi $Na_2[^{51}Cr]O_4$ (1 mCi/ml) was added to each culture dish. Values are means of triplicate cultures.

† Counts were reduced significantly from control value ($P < 0.02$).

‡ Percentage inhibition was calculated as described in Materials and Methods.

§ No statistical significance.

Table 2. Inhibition of macrophage ingestion of ^{51}Cr-labeled SRBC by tetracaine

Treatment concentration	SRBC condition	Average ^{51}Cr-activity in lysate at 2 hr* (cpm ± S.D.)
None, CMEM	Opsonized	7672 ± 730
Tetracaine (100 μM)	Opsonized	4828 ± 371† (37)‡
None, CMEM	Unopsonized	1613 ± 122
Tetracaine (100 μM)	Unopsonized	1058 ± 240§ (35)‡

* Macrophage cultures were incubated for 2 hr in 100 μM tetracaine or CMEM after which the phagocytosis assay was incubated in CMEM; 0.2 ml of a 2% opsonized or unopsonized SRBC suspension, labeled with 200 μCI $Na_2[^{51}Cr]O_4$ (1 mCi/ml) was added to each culture dish. Values are means of triplicate cultures.

† Counts were reduced significantly from control value ($P < 0.02$).

‡ Percentage inhibition was calculated as described in Materials and Methods.

§ Counts were reduced significantly from control value ($P < 0.05$).

100 μM tetracaine reduced the recognition of opsonized SRBC by 53 per cent ($P < 0.02$), whereas the recognition of unopsonized SRBC was not altered significantly ($0.2 < P < 0.5$). Inhibition of phagocytosis of opsonized SRBC by tetracaine, therefore, appears to be due, in part, to impairment of recognition.

Inhibition of SRBC ingestion. Having established that opsonin recognition was sensitive to tetracaine while unopsonin recognition was not, we sought to determine whether ingestion was affected similarly. In this experiment, macrophage monolayers were preincubated for 2 hr at 37° with either CMEM or CMEM containing 100 μM tetracaine. The cultures were rinsed, overlaid with ^{51}Cr-labeled SRBC suspended in CMEM, and incubated at 37° for an additional 2 hr. The data indicate (Table 2) that untreated macrophages ingested approximately five times as many opsonized as unopsonized SRBC. Tetracaine treatment reduced the ingestion of opsonized SRBC by 37 per cent and the ingestion of unopsonized SRBC by 35 per cent.

The similarity in inhibition of both types of SRBC by 100 μM tetracaine prompted us to investigate the dose–response of inhibition of opsonized and unopsonized SRBC to tetracaine treatment. The relation of per cent inhibition to tetracaine concentration is shown in Table 3. The least squares linear regression for opsonized SRBC is described by the equation $Y = -43.0 + 41.8 \log_{10}$ (1000X). The corresponding equation for unopsonized SRBC is $Y = -33.2 + 29.5 \log_{10}$ (1000X). A *t*-test for parallelism indicated that the regression lines are not parallel ($P < 0.05$).

Recognition of mouse IgG. Inhibition of immune recognition and ingestion appeared to be separable steps, based on the above observations that tetracaine could inhibit immune but not non-immune recognition and that ingestion was sensitive to tetracaine regardless of the mode of recognition. What then was the basis of inhibition of immune recognition? To study this, the following recognition experiment was performed. Purified, heat aggregated, ^{125}I-labeled mouse IgG was incubated for 20 min under the same conditions as described for the whole cell regognition experiment above. Macrophages pretreated for 2 hr with 100 μM tetracaine took up to 5800 ± 1800 cpm, presumably by their Fc receptors. The corresponding value for untreated macrophages was 4900 ± 1200 cpm. These values are not significantly different from each other ($0.2 < P < 0.5$).

DISCUSSION

Evidence is accumulating which points to the active participation of the Fc receptor on the macrophage surface in attachment of the opsonizing antibody during phagocytosis [12–14]. We have observed in a previous study that the phagocytosis of opsonized SRBC

Table 3. Dose–response of inhibition of ingestion

Tetracaine concentration (μM)	Opsonized Average activity in lysate* (cpm ± S. D.)*	% Inhibition†	Unopsonized Average activity in lysate* (cpm ± S. D.)	% Inhibition†
0	7672 ± 730		968 ± 217	
10	8505 ± 357	0	1034 ± 289	0
50	7039 ± 492	8	789 ± 78	18
100	4828 ± 371	37	863 ± 206	11
500	2756 ± 442	64	620 ± 190	36
1000	757 ± 168	91	409 ± 94	58

* Conditions were as given in the legend to Table 2 and the text.

† Per cent inhibition was calculated as described in Materials and Methods.

by mouse peritoneal macrophages *in vitro* could be inhibited by tertiary amine local anesthetics. This inhibition is reversible in a concentration-dependent fashion [1]. The endpoint of those observations was the uptake of ^{51}Cr-labeled opsonized SRBC by untreated and local anesthetic-treated macrophages after a 2-hr interaction. Those experiments, therefore, could not distinguish whether immune recognition was inhibited or the ingestion mechanism was paralyzed or both.

We have shown that immune recognition of opsonized SRBC could be inhibited by 100 μM tetracaine, while non-immune recognition of unopsonized SRBC could not. This suggests that tetracaine exerts its effect, in part, by acting on the Fc receptor. The dose–response relationships indicate that the ingestion step is also sensitive to tetracaine treatment. That the dose–response slope for opsonized SRBC is steeper than that for unopsonized SRBC suggests that tetracaine can exert its effects directly on the attachment of the macrophage Fc receptor to the Fc of the opsonizing antibody. We have already ruled out the possibility that tetracaine acts on the antibody directly [1].

The inhibition of phagocytosis of opsonized SRBC with local anesthetics could represent the disruption of membrane associated microtubles and microfilaments involved in the transmembrane control of the Fc receptor, thereby altering its presentation on the macrophage surface. In fact, our original study demonstrated that the extent to which phagocytosis of opsonized SRBC was inhibited by tertiary amine local anesthetics was proportional to their affinity for lipid [1]. This is similar to the cytoskeletal transmembrane control of lymphoid surface receptors and the effect of local anesthetics on the mobility and distribution of these receptors [3, 4].

If the presentation of the Fc receptor on the macrophage surface is altered, it could be accomplished in four ways: internalization, extrusion, reorientation into the membrane lipid bilayer, or redistribution as in clustering. The first three possibilities are excluded by the recognition of the ^{125}I-labeled, aggregated IgG experiment. Those alternatives would result in a reduction in ^{125}I-binding. That no such reduction was observed strongly suggests that clustering of receptors occurred. That is, under conditions where phagocytosis of opsonized SRBC was reduced by 37 per cent and recognition of whole cells was reduced by 53 per cent, binding of aggregated IgG was unimpaired. It is easy to envision how clustering of receptors could account for decreased recognition of large SRBC by steric hindrance while at the same time having no effect on recognition of small IgG aggregates. When taken together with the slope differences of Table 3, the data suggest that the presentation of the Fc receptor is important during both the recognition and ingestion steps.

We have shown, then, that tetracaine-induced inhibition of immune-mediated phagocytosis involves both redistribution of Fc receptors and paralysis of the ingestion step. The precise nature of the effect of tetracaine on ingestion is not clear. There is ample evidence of the physical disruption of cytoskeletal elements by tertiary amine anesthetics [2–4]. Physical disruption of the macrophage cytoskeletal system would result in a handicapping of macrophage phagocytic movement and the inability to engulf particulate objects at the post-recognition stage.

It is interesting that lidocaine-induced inhibition of phagocytosis by human leukocytes is accompanied by decreased oxygen consumption [15]. This suggests that the post-recognition disruption of cytoskeletal elements may, in fact, be at the level of energy metabolism.

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