# USE OF A FLUOROCHROMED CULTURE OF MACROPHAGES TO DETERMINE ACTIVITY OF ANTILYSOSOMAL SERA

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A method of quantitative determination of the activity of antilysosomal sera based on their action on the red cytoplasmic granules (lysosomes) of albino mouse macrophages fluorochromed intravitally with acridine orange is proposed. On contact between 4- to 5-day cultures of macrophages fluorochromed with acridine orange and antilysosomal sera in the presence of complement from macrophage cytoplasm the red granules were shown to disappear and the morphology and luminescence of the nuclei are changed. Similar changes are observed if contact between macrophages and antisera takes place before fluorochroming with acridine orange. Disappearance of the granules does not take place through contact between macrophages with normal rabbit serum in the presence of complement or contact with antilysosomal sera in the absence of complement. The ratio between the numbers of normal and changed cells depends on the dilution of the sera and can act as a quantitative criterion for assessing activity of the serum.

Data published in recent years indicate that the characteristics of the membranes and enzyme systems of the macrophagal lysosomes may play an important role in the natural resistance of the body and in the initial stages of antibody formation [5, 6, 9, 10]. To study the role of lysosomes in these processes, antisera against whole lysosomes and their components are used. Activity of antilysosomal sera is usually determined by the use of various serological reactions [1, 12, 15, 16], including luminescence-serological tests [13]. However, these methods of estimating the activity of antilysosomal sera are indirect; they cannot be used to judge the character of action of these sera on the lysosomes of living cells, nor can they be used for the quantitative evaluation of that action.

The action of antilysosomal serum is judged on the basis of the cytochemical reaction for acid phosphatase [16]. However, this method is complicated and is not quantitative. When the cytotoxic action of antilysosomal sera is determined on polymorphs and other cells by phase-contrast microscopy [12, 16], besides lysosomes other intracellular structures are detected and the cytotoxic action may not depend only on antilysosomal activity. Determination of the activity of antilysosomal sera by their granulolytic action on isolated liver lysosomes and polymorphs requires a complex procedure of isolation of the lysosomal fraction and biochemical determination of the enzymes, and it cannot shed light on the character of their action on the living cell [12, 14].

The object of this investigation was to develop a technique which can be used to estimate the activity of antilysosomal sera by their action on the membrane and function of lysosomes in the living cell.

For this purpose a culture of albino mouse peritoneal macrophages, fluorochromed intravitally with acridine orange (AO), was used, for under those conditions red cytoplasmic granules (RCG) which, as some authors have shown [2, 8], are lysosomes can be observed in the cells.

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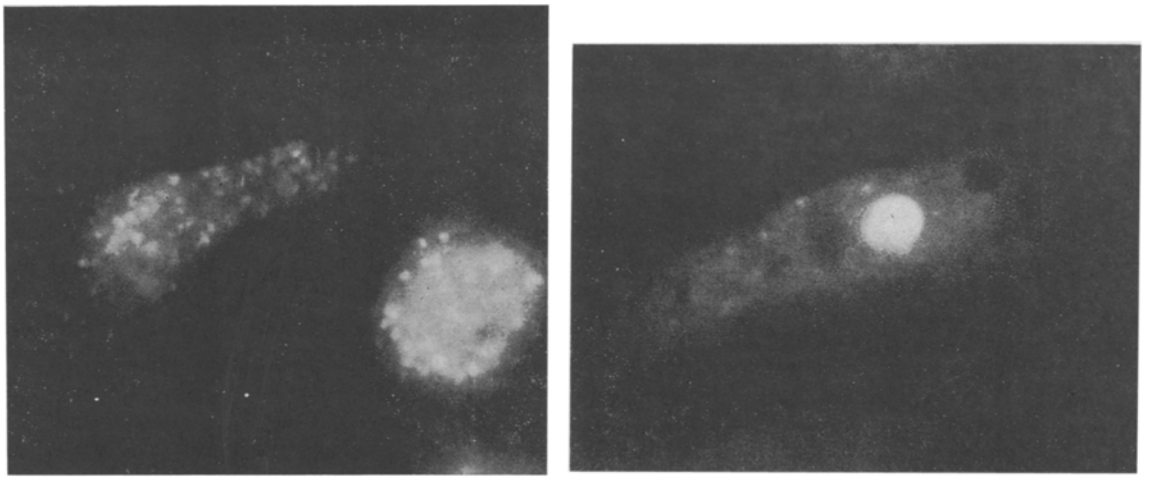

Fig. 1. Fluorochromed macrophages. Left, not treated with antisera: red cytoplasmic granules can be seen; right, treated with antimembrane serum; red cytoplasmic granules absent. ML-2 luminescence microscope, objective 90 × (MI), Homal 3 ×.

## EXPERIMENTAL METHOD

To obtain antisera, various subcellular fractions of spleen cells from mice of the A strain were injected at weekly intervals intramuscularly into rabbits three times with Freund's complete adjuvant in the following mean quantities per injection: lysosomes 10 mg protein, lysosomal enzymes 20 mg protein, lysosomal membranes 30 mg protein, microsomes 25 mg protein, pH 5-enzymes 45 mg protein. Protein was determined by Lowry's method. The serum was obtained on the 10th day after the third injection of antigen and was heated to 56°C for 30 min.

Subcellular fractions were obtained from spleen cell homogenates by differential centrifugation; lysosomes by the method of Weissmann and Thomas [18] and pH 5-enzymes and microsomes as described previously [7].

Peritoneal macrophages were obtained from unstimulated albino mice by washing out the peritoneal cavity with cold Hanks's solution containing heparin (5 units/ml). The suspension of cells ($2 \cdot 10^6$) in a volume of 2 ml was poured into test tubes with coverslips and incubated for 1 h at 37°C to produce attachment of the cells, after which those remaining unattached to the glass were washed off with medium No. 199 containing 10% inactivated bovine serum (BS). The culture was incubated in the same medium.

The macrophages were fluorochromed intravitally with AO by the method used by Korn and Grigor'eva [3, 4]: a solution of AO was added to the culture of macrophages to a final concentration of 2 $\mu$g/ml and the culture was then incubated for 30 min at 37°C. It was then washed twice with the same medium without AO and allowed to stand in that medium for 18 h at 37°C.

The cells were then treated with antisera and examined in the ML-2 luminescence microscope.

In some experiments the cultures were fluorochromed (by the same method but without washing for 18 h) after treatment with antisera.

A culture (3-4 days) of albino mouse peritoneal macrophages (on coverslips) was incubated for a certain time (2-3 h at 37°C) with various dilutions of antilysosomal serum which had been heated to 56°C for 30 min with the addition of previously titrated guinea pig complement, usually in a dilution of 1:10 (standard lyophilized complement). Incubation was carried out in medium No. 199 with 10% BS. The control consisted of fluorochromed macrophages: 1) not treated with antisera, but with the addition of complement, 2) with the addition of normal rabbit serum (NRS) and complement, and 3) with the addition of antiserum but without complement.

After incubation the macrophage cultures on the coverslips were washed off three times with medium No. 199 containing 10% BS. They were placed with the cells underneath on a slide, leaving a capillary space filled with medium No. 199, and they were then examined in the ML-2 luminescence microscope.

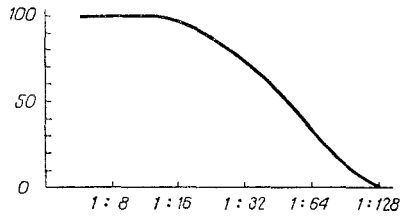


Fig. 2. Number of macrophages without RCG as a function of dilution of antilysosomal serum. Abscissa, dilutions of antimembrane serum; ordinate, percentage of macrophages without RCG.

TABLE 1. Percentage of Macrophages Not Containing RCG after Contact with Different Dilutions of Antilysosomal Antimembrane Serum

| Samples | Cells without RCG |
|---|---|
| Control: without antiserum, but with complement | 0 |
| with NRS and complement | 0 |
| with antiserum, without complement | 0 |
| Experiment: dilutions of antimembrane serum: | |
| 1:8 | 100 |
| 1:16 | 100 |
| 1:32 | 75 |
| 1:64 | 35 |
| 1:128 | 0 |

## EXPERIMENTAL RESULTS

Examination of the control preparations under high power (objective 90, ocular 7) of the luminescence microscope revealed cells with typical morphology, with a dull green nucleus, brighter luminescent nucleoli, and palely luminescent green cytoplasm, filled with bright red cytosplasmic granules chiefly in the perinuclear region (Fig. 1A). The chief distinguishing feature of cells incubated with antiserum against lysosomal membranes in the presence of complement was absence of these RCG in their cytoplasm (Fig. 1B). Besides these changed cells, normal cells indistinguishable from the control could also be found in the preparations. These results were observed in preparations treated with antisera both before and after fluorochroming. The ratio between the numbers of the two types of cells depended on the dilution of antiserum (Table 1). Cells containing RCG (red) and cells not containing RCG (green) could be counted under the low power of the microscope (objective 10, ocular 7).

It is best to take the dilution at which the number of cells without RCG was 50% as the titer of the serum. This dilution can be determined from the calibration curve (Fig. 2).

Similar results were obtained when the activity of antisera against whole lysosomes and lysosomal enzymes was determined. The changes in morphology and luminescence differed somewhat after treatment with antisera against the other subcellular fractions, viz., pH 5-enzymes and microsomes: pink luminescence of the cytoplasm and solitary yellow and green granules were observed. The partial granulolytic action of these sera on the RCG could be the result of their containing antibodies against antigens common to lysosomes.

The conclusion to be drawn from these results is probably that antilysosomal sera have a specific lytic action on the lysosomal membrane, which was observed only in the presence of complement, thus indicating the immunological character of the reaction [13, 14]. Meanwhile this is additional proof of the lysosomal nature of the RCG in the macrophages. Changes in the morphology and character of luminescence of the macrophagal nuclei are evidently secondary and take place through the influence of enzymes liberated by lysis of the lysosomal membranes.

The arguments contained in the paper by Weiss [17] on the possibility of an indirect action of antilysosomal sera are based on observations that these sera have no lytic action on lysosomes isolated from the cell. However, the work of Trouet [13], Tulkens et al. [14], and Quie [12] has shown that antilysosomal sera possess such a lytic action. The statement that antibodies against endoplasmic reticulum are active is also unconvincing in view of the discovery [13] that the endoplasmic reticulum and lysosomal membrane have common antigens.

Persellin [11] found a stabilizing action of antilysosomal sera on the lysosomal membrane which he explains on the grounds that the serum he obtained had antibodies only against membranes and not against other components of the lysosomes. However, these results were not confirmed by other investigators working with highly specific sera against individual components of the lysosomes [12, 13].

The evidence put forward by Trouet [13] of the nonspecificity of the stabilizing action of the antilysosomal serum found by Persellin [11] can be accepted. One piece of evidence of this nonspecificity is that this action is independent of complement.

The mechanism whereby antilysosomal sera penetrate into the living cell is not yet fully understood: do they penetrate by pinocytosis or is there a change in the permeability of the cell membrane but without its undergoing lysis, under the influence of these sera? Tulkens et al. [14] showed that antienzyme sera

penetrate into lysosomes by pinocytosis, and they then inhibit the activity of the lysosomal enzymes. The data in that paper seem more convincing than the results obtained by Weiss [17], which he takes as evidence of a change in the permeability of the cell membrane. Any such change would be more likely to be secondary, brought about by the enzymes liberated from the lysosomes.

## LITERATURE CITED


1. L. G. Zaitseva and M. E. Bubashvili, in: Cytotoxins in Modern Medicine [in Russian], No. 6, Kiev (1972), p. 124.
2. A. V. Zelenin, Izvest. Akad. Nauk SSSR. Seriya Biol., No. 6, 977 (1969).
3. M. Ya. Korn and M. A. Grigor'eva, in: Proceedings of the Fifteenth All-Union Congress of Microbiologists, Epidemiologists, and Infectious Diseases Specialists [in Russian], No. 12, Moscow (1970), p. 161.
4. M. Ya. Korn, Abstracts of Proceedings of an All-Union Conference on Luminophores [in Russian], Stavropol' (1971), p. 67.
5. I. Ya. Uchitel' and É. L. Khasman, Vestn. Akad. Med. Nauk SSSR, No. 10, 228 (1969).
6. I. Ya. Uchitel', Vestn. Akad. Med. Nauk SSSR, No. 7, 65 (1970).
7. I. Ya. Uchitel' and É. L. Khasman, Biokhimiya, No. 1, 13 (1967).
8. A. C. Allison and M. R. Young, Life Sci., 3, 1407 (1964).
9. D. Campbell and J. Garvey, Adv. Immunol., 3, 261 (1963).
10. Z. Cohn and B. Ehrenreich, J. Exp. Med., 129, 201 (1969).
11. R. H. Persellin, J. Immunol., 103, 39 (1969).
12. P. Q. Quie and J. G. Hirsch, J. Exp. Med., 120, 149 (1964).
13. A. Trouet, Caractéristiques et Propriétés Antigéniques des Lysosomes du Foie, Brussels (1970), p. 29.
14. P. Tulkens, A. Trouet, and F. van Hoff, Nature, 228, 1282 (1970).
15. L. Weiss and J. T. Dingle, Ann. Rheum. Dis., 23, 5763 (1964).
16. L. Weiss, Cell Res., 37, 540 (1965).
17. G. Weissmann and L. Thomas, J. Exp. Med., 116, 433 (1962).
18. G. Wiedermann, H. Denk, and D. Kraff, Z. Immun. Exp., 141, 322 (1971).