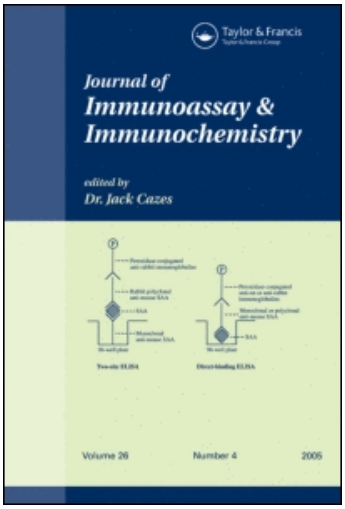

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An Automated Micromethod for Detection of Lytic Antimycobacterial Antibodies by Immune Lysis of Liposomes Sensitized to Tuberculin

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AN AUTOMATED MICROMETHOD FOR DETECTION OF LYTIC ANTIMYCOBACTERIAL ANTIBODIES BY IMMUNE LYSIS OF LIPOSOMES SENSITIZED TO TUBERCULIN

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ABSTRACT

Calcein was concentrated inside multilamellar liposomes (MLV) by encapsulation through an osmotic gradient in order to increase its quenching. Immune lysis of these MLV sensitized to PPD by direct encapsulation or by exposure to preformed vesicles was studied in the presence of rabbit anti-BCG serum and of a non-immune rabbit serum as a source of complement, successively added in the wells of a microplate to a final volume of 0.1 ml. A 40 to 50 % release of encapsulated calcein was observed after 5 min using a Titertek Fluoroskan II. Preformed liposomes exposed to PPD were more sensitive to anti-BCG serum than liposomes formed in PPD. Trials with human sera revealed a significantly higher level of specific lytic immunoglobulins in tuberculous patients than in non-tuberculous subjects.
Key words : Liposome immune lysis assay; lytic antimycobacterial antibodies; tuberculosis, human; test automation

INTRODUCTION

Complement dependent immune lysis assay of liposomes entrapping fluorescent marker and sensitized to tuberculin

(PPD) was developed recently by Legros et al (1,2). This method raised two technical problems. First, it was performed in two steps; the immune lysis occured in a test tube and fluorometric reading was performed on aliquots sampled from this tube using a conventional spectrofluorimeter. This condition restricted the number of samples which could be measured in one day. Second, the large reaction volume led to the use of relatively large amounts of sensitized liposomes and of serum to be tested.

We have developed an automated micromethod allowing a direct reading in a small reaction volume, using a Titertek Fluoroskan II. Multiple tests were performed and samples were assayed in 96-well microplates, as previously developed by Ishimori et al (3). The method was elaborated with liposomes sensitized to tuberculin, anti-BCG serum and rabbit serum as a source of complement. Kinetics and sensitivity of immune lysis were evaluated. Trials were performed on serum from tuberculous and non-tuberculous patients.

MATERIALS AND METHODS

Buffer

The medium used in the immunoassays was a Tris-HCl buffer, pH 7.4, brought to selected osmolalities by adding NaCl (TBS). Osmolalities were measured using a Fiske Osmometer (Fiske Associates, Burlington, MA).

Lipids

Egg phosphatidylcholine (Egg Pc), 100 mg/ml in a chloroform solution, and cholesterol were purchased from Sigma(St Louis,

MO). Cholesterol was dissolved at a concentration of 10 mg/ml in chloroform. Both lipids were stored at -18°C.

Immunological Reagents

Tuberculin (Purified Protein Derivative : PPD) from Mycobacterium bovis (Instituut Pasteur van Brabant, Brussels) was prepared by the method of Magnusson and Bentzon (4). Proteins of a heat-killed BCG (Bacillus Calmette-Guérin) culture filtrate were precipitated by 2 % trichloroacetic acid. The precipitate was successively washed with acetone and anhydrous ether. The product was dried to a powder and stored at 4°C.

A rabbit antiserum directed against BCG was purchased from Dakopatts (Copenhagen, Denmark). A sonicate, containing soluble and insoluble antigens from BCG, had been used for immunization. A detailed description of the antiserum's characteristics is found in Closs et al (5).

Rabbit sera which were previously proved not to induce immune lysis of liposomes sensitized to PPD were used as an exogenous source of complement.

Other reagents

2', 7'-bis-(N,N'-di-(carboxymethyl)aminomethyl) fluorescein (calcein) (Sigma) was dissolved in 1 M NaOH as recommended by Allen and Cleland (6). Osmolality was brought to 90 or 295 mOsm/Kg H2O (mOsm) with TBS; the final concentration of calcein was 15 mM in both cases.

Studies of Liposomal Calcein Quenching

Chloroform was evaporated under a nitrogen atmosphere from a solution containing 20 mg of lipids (0.144 ml Egg pc and 0.56

ml cholesterol; molar ratio 4:3). After drying under vacuum for 4 h, calcein prepared as described above was encapsulated inside the MLV by adding 1 ml of the fluorescent probe solution to the lipid film and vortexing. Equiosmolar conditions were obtained by forming liposomes in calcein 295 mOsm. After 1 h at room temperature, unencapsulated calcein was removed by 4 successive centrifugations (10 min, 5000 rpm) in 1 ml TBS at the same osmolality. Alternatively, an osmotic gradient was created by encapsulating calcein 90 mOsm and washing 4 folds in TBS 295 mOsm.

Preparation of Liposomes Sensitized to Tuberculin

Lipid films were obtained as described above. Sensitization of liposomes to tuberculin was performed by two procedures : (i) formation of liposomes in a calcein-PPD solution or (ii) exposure of preformed liposomes containing calcein to PPD.
In the first technique, 0.375 ml of the calcein solution (15mM 90 mOsm) containing 2 mg/ml PPD were added to 20 mg of lipid film. After vortexing, the liposomes were kept for 1 h at room temperature in the dark and centrifuged 4 times for 10 min at 5000 rpm in 295 mOsm. The liposomal pellet was resuspended in 0.375 ml TBS so that the final lipid concentration was 53 mg/ml.

Alternatively, liposomes were formed in 0.375 ml calcein (15mM, 90 mOsm). After 1 h, unencapsulated calcein was eliminated by 4 washes in TBS 295 mOsm. The liposomal pellet was suspended for 1 h in 0.375 ml TBS 295 mOsm containing 2 mg/ml PPD. After 4 washes in TBS at the same osmolality, liposomes were resuspended in 0.375 ml.

Immune Lysis Assay

The following reagents were added in the wells of a microplate (Nunc, Roskilde, Sweden). Rows A and B : 0.015 ml of the liposome-calcein preparations, unsensitized (row A) or sensitized (row B) to PPD, and 0.085 ml rabbit serum. Rows C to H : 0.015 ml of liposomes sensitized to PPD, 0.012 ml anti-BCG or patient's serum and 0.073 ml rabbit serum.

Fluorescence was measured using a Titertek Fluoroskan II (Flow Laboratories, Mc Lean, Virginia). Excitation was at 490 nm and emission at 520 nm. This Fluoroskan allows the measurement of the fluorescence of one well in 0.1 sec. Sustained reading of a 96 well microplate thus lasted about 10 sec and results were immediatly printed out. Results can be expressed in absolute fluorescence units (A.F.U.) or in relative fluorescence (R.F.)choosing one experimental condition as 100 %. Row A was used to blank the Fluoroskan on calcein quenched inside the liposomes. This level of fluorescence was automatically substracted by the Fluoroskan for the final expression of results. 0.01 ml 10 % deoxycholic acid was added to the wells of row B in order to release all the entrapped calcein in the raction medium. Using the R.F. mode of the Fluoroskan, the mean fluorescence of the wells of row B defined 100 % liposomal lysis. Percent of specific immune lysis in rows C to H, expressed as (A.F.U. of immune lysis conditions / A.F.U. of row B) X 100, was directly indicated by the Fluoroskan. At the end of each experiment, 0.01 ml 10 % deoxycholic acid were added to the wells of rows C to H and A.F.U. was controled to be statistically identical to row B.

Human Assays

Leakage of calcein out of the preparations of PPD-sensitized liposomes and out of the preparations of unsensitized MLV was measured after 75 min in the presence of human sera from 10 tuberculous patients and from 10 healthy subjects. Proportions of sera, liposome preparations and non-immune rabbit serum were the same as in the foregoing assays, anti-BCG serum being replaced by the patient's serum to be tested. Results were expressed as the mean $\pm$ SD of values obtained with three batches (N=3). Statistical significance on the mean value of the two groups of patients was determined by Student's t test.

Human sera were obtained from the Erasme University Hospital (Brussels, Belgium). Tuberculous patients had cavitating lesions on chest X-ray and were sputum positive by direct assay. They were different from patients tested in the previous study (2). Sera were sampled at the time of diagnosis and before treatment. Healthy subjects were all tuberculin skin test negative.

Two sera of tuberculous patients and two sera of non-tuberculous subjects inducing a calcein release averaging the mean lysis of the two series were tested at dilutions from 1/8 to 1/500 using 3 preparations of liposomes sensitized to tuberculin. Results were pooled for the two tuberculous and the two non-tuberculous sera and Means $\pm$ SD were calculated.

RESULTS

Liposomal Calcein Quenching

The maximal fluorescence reading capacity of the Fluoroskan was limited to 25 to 30.10^3 A.F.U.. In our experimental condi-

tions, this must correspond to the maximal amount of calcein entrapped into 0.015 ml of liposomes in order to ensure an optimal measurable fluorescence after complete lysis by deoxycholic acid in a final volume of 0.11 ml. Such a level of entrapped calcein was obtained when liposomes (20 mg/ml) were formed in 1 ml calcein 15 mM and diluted to 5 mg/ml.

Allen (7) has shown that self-quenching of calcein depends on its concentration inside liposomes, i.e. the more the calcein is concentrated, the more it is quenched. The optimal use of the liposome immune lysis method should thus aim at obtaining the highest concentration of calcein quenched inside the vesicles in order to increase the difference of A.F.U. between quenched and totally released calcein. Calcein 15 mM, 90 mOsm in TBS, was entrapped into liposomes which were further washed and suspended in 295 mOsm TBS. In such a case, an osmotic gradient was created between the outer phospholipid bilayer. Calcein was consequently concentrated and thus more quenched inside liposomes when compared to encapsulation and washes in 295 mOsm equiosmolar conditions. The osmotic gradient thus resulted in decreasing the liposomal fluorescence, allowing the reduction of the blank value from 9.5 to 5.5 $.10^{3}$ A.F.U. Calcein encapsulated in both osmotic conditions did not spontaneously leak from the liposomes in detectable amounts for at least 3 h. The ratio dequenched / quenched fluorescence (quenching ratio) raised from 2.7 to 4.5 when calcein was entrapped following the osmotic gradient. For further use in immune assays, calcein was concentrated inside MLV by encapsulation through the osmotic gradient.

Time Course of the Reaction

When liposomes sensitized to PPD were incubated with anti-BCG rabbit serum and non-lytic rabbit serum as a source of complement, the level of calcein release rapidly increased, reaching a plateau (45-50 % of the total entrapped probe measured in the presence of deoxycholic acid) after 5 min and remaining unchanged for another 70 min (Fig. 1). Statistically comparable results were obtained with both types of PPD sensitized liposomes. Ten preparations of each type of MLV were tested. Standard deviations did not exceed 10 % of the mean values.

Titration of the Anti-BCG Antiserum

Liposomes formed by direct encapsulation of PPD or by exposure of preformed vesicles to a PPD solution were exposed to anti-BCG serum at up to 2000-fold dilutions. Immune lysis was measured after 75 min. The percentage of immune lysis decreased exponentially for both types of sensitized liposomes (Fig. 2). This decrease was more rapid for liposomes encapsulating PPD than for preformed MLV exposed to PPD. Four preparations of each type of MLV were tested and Means $\pm$ SD were calculated. Standard deviations were low for preformed liposomes, being inferior to 10 % of the mean values.

Assays on Human Sera

Preformed liposomes exposed to PPD and MLV unsensitized to PPD were incubated in human sera from 10 tuberculous and 10 non-tuberculous patients. Individual percentages of lysis after 75 min are shown in Table 1. No leakage of calcein from

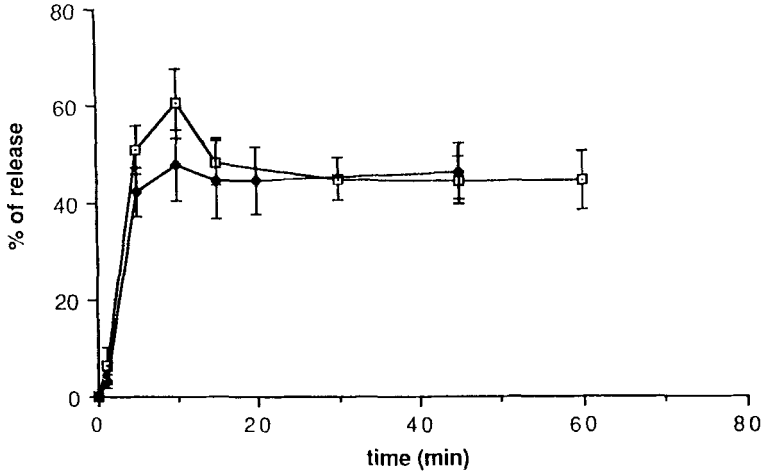

Figure 1 : Time course of calcein release obtained when liposomes formed in BCG PPD (□) or preformed liposomes exposed to BCG PPD (●) were incubated with anti-BCG serum and rabbit serum as a source of complement. Mean ± SD, n=10

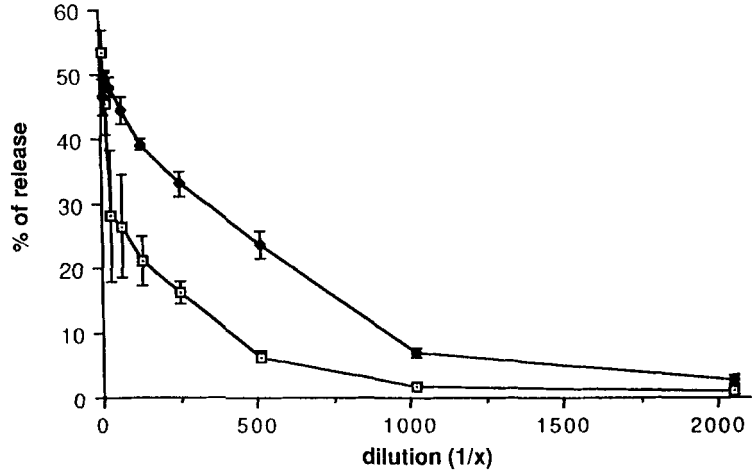

Figure 2 : Effect of anti-BCG serum dilution on liposome immune lysis , (□) encapsulation of PPD; (●) preformed liposomes exposed to PPD. Mean ± SD, n=4

TABLE 1

Percent of Encapsulated Calcein Release in Tuberculous and Non-Tuberculous Human Sera.

Tuberculous Patients			Non-Tuberculous Patients		
Number	L.PPD[a]	L.[b]	Number	L.PPD[a]	L.[b]
1	6.8±2.3	0	1	2.9±1.1	0
2	19.0±1.4	0	2	2.1±1.6	0
3	16.0±0.5	0	3	2.3±0.9	0
4	15.0±0.4	0	4	3.4±0.5	0
5	24.0±1.5	0	5	0.0±0.0	0
6	20.0±2.1	0	6	0.5±0.8	0
7	31.6±1.0	0	7	1.2±1.3	0
8	6.4±0.3	0	8	0.0±0.0	0
9	14.7±1.3	0	9	0.6±0.6	0
10	20.5±0.8	0	10	0.0±0.0	0

a. Incubation with Liposomes formed in BCG/PPD Solution (L.PPD) and
b. with Liposomes without PPD (L.).
c. Statistical Significance determined by Student's t test ($p<0.01$). Mean ± SD, N=3)

unsensitized liposomes was observed in the 2 series of patients, both tuberculous and non-tuberculous. The immune lysis of liposomes sensitized to PPD was statistically higher in sera of tuberculous than of non-tuberculous patients ($p<0.01$).

The titration curves of 2 sera from tuberculous patients and of 2 sera from healthy subjects indicated that the mean liposome lysis exponentially decreased in the presence of tuberculous sera, while it did not significantly vary for non-tuberculous sera. For the tuberculous patients, immune lysis levels significantly higher than the ones induced by healthy subjects were still observed at a dilution of 1/32.

DISCUSSION

Although it is known that immunological defenses against mycobacterial infections are mainly attributed to cell-mediated immunity, detection of antimycobacterial antibodies represents a tool for laboratory diagnosis of tuberculosis (8-11). A limited number of studies has been devoted to the role of lytic antibodies due to the lack of specific, trustable and easy-to-handle methods of detection (For a review, see ref.10).

The present study demonstrated that stable and non-permeable liposomes encapsulating calcein through an osmotic gradient and sensitized to PPD were able to detect lytic antimycobacterial antibodies in the sera of tuberculous patients. Features of the method deserve some comments.

Two modes of sensitization of liposomes to tuberculin have been applied, either direct encapsulation or exposure to preformed vesicles. Kinetics of immune lysis were identical in both cases and reproducible from one preparation to another. Maximal lysis was obtained after 5 min., thus ensuring the rapidity of the immune reaction and of the test. The maximal percentage of calcein release was identical to the one reported by Richards et al (12).

Titration curves of the two types of PPD-sensitized liposomes indicated that affinity for antibodies from a calibrated anti-BCG antiserum was reproducible between different batches. The decrease of the antiserumdilution curve was sharper for liposomes encapsulating PPD than for preformed vesicles exposed to tuberculin. This can be explained by a better immunoreactivity of antigens when they were added to

preformed MLV previously submitted to an osmotic gradient, opposite to what has been previously observed in equiosmolar conditions (2).

Both types of MLV proved to be very stable in Tris buffer as well as in human sera.

Assays on human sera indicated that samples from tuberculous patients contained higher levels of lytic antibodies against PPD antigens than samples of non-tuberculous subjects. Presence of lytic antibodies in sera of non-tuberculous patients seems rather astonishing at first glance, but it must be recalled that Turneer et al (13) also detected low levels of antibodies against PPD in sera of non-tuberculous, skin test negative subjects, using enzyme immunoassays. In addition, titration curves of tuberculous sera exhibited an exponential decrease with a specific immune lysis at a dilution up to 1/32, which was not the case for sera of non-tuberculous subjects.

Results obtained with our liposome immune lysis assay are difficult to compare with other immunoassays such as radioimmunoassays, fluorescent assays or enzyme assays (for review see ref.10), since our method only detects lytic immunoglobulins. When comparing with previous results obtained in a tube format reading aliquots in a conventional spectrofluorimeter (1,2), the quantitative interassays results obtained here are more reproducible.

The clinical significance of lytic antibodies in the tuberculous disease may not be drawn from our present limited results. But the liposome immune lysis assay has now been elaborated as an automated micromethod allowing to test rapidly and routinely a large number of samples, thus opening the way

to an extensive study of lytic antibodies evolution during the tuberculous disease.

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