*Immunogical Study of Anti-Beta-Lactamase Antibodies by Acidimetric Methods

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A beta-lactamase was extracted from an *Escherichia coli* K-12 strain carrying the R-TEM plasmid and has been purified by affinity chromatography. Antisera to this enzyme were prepared in the rabbit, and the enzyme-antibody neutralization reaction has been evaluated with acidimetric methods (pH stat or pH meter). Under defined experimental conditions, it is now possible to clearly illustrate the enzyme-antibody reaction by means of accurate, rapid, and simple-to-perform methods. These methods are in accord with the specificity of the two reactive partners and allow the detection of the enzyme in a crude bacterial extract which would eventually contain more than one beta-lactamase.

Since several years ago, many workers have been dealing with the identification and characterisation of beta-lactamases produced by different gram-negative bacteria (2, 3, 4, 5, 8, 9, 10, 14, 15, 16). These studies are highly important because this class of enzyme is often implicated in chemotherapy. In fact, there is a rapid increase in the number of bacterial strains resistant to penicillins or cephalosporins, and it has been shown that production of beta-lactamase is the principal mechanism of such resistance.

Until now, nobody has dealt with the epidemiological aspect of the frequency and the distribution of the different beta-lactamases. This fact could be explained in part by the lack of rapid and reliable methods of detection and identification of beta-lactamases.

We have recently developed a new microacidimetric computerized method (7) which allows highly precise determination of the enzymatic activity spectrum (K_m and V_{max}).

This paper deals with the application of this method to the identification of a beta-lactamase by use of anti-beta-lactamase antibodies. The production of antisera which react with beta-lactamase gains nothing in originality. It has already been shown by other investigators (3, 5, 8, 11, 12, 13, 14, 15) that specific antibody can neutralize the enzyme reaction and neutralization tests have led to classification of beta-lactamases of gram-negative bacteria into different classes (14, 15).

Here again, except for the usual immunological methods of detecting antigen-antibody complexes such as immunoelectrophoresis (15), the manometric (11, 12) or iodometric (5) method commonly used to verify the neutralizing power of antibodies is not easily performed. A critical study of these methods has already been made (6) and their lack of accuracy, reliability, and specificity has been discussed.

Our present investigation describes the production and activity of anti-beta-lactamase antibodies and their application to the identification of the enzyme. The TEM beta-lactamase was chosen because of its wide distribution and also because it is a particularly well known reference enzyme.

MATERIAL AND METHODS

Bacterial strains. The beta-lactamase is extracted from an *Escherichia coli* K-12 (carrying the R-TEM plasmid). The two strains used to control the antiserum specificity were: (i) *E. coli* P 453, type II (17; a wild-type ampicillin-resistant strain producing a beta-lactamase that is kinetically very close to the TEM beta-lactamase); (ii) *Pseudomonas aeruginosa* Marmot (penicillin and cephalosporin resistant; R. Labia, unpublished data). This strain produces two distinct beta-lactamases which can be separated by affinity chromatography. The profile obtained by the computerized microacidimetric method and the determination of the isoelectric point (pI) shows that one beta-lactamase is the TEM (pI = 5.40) and that the other one is a cephalosporinase-like beta-lactamase (pI = 9.20).

Preparation of the crude enzymatic extract. Bacterial culture was grown routinely at 37 C in tryptone broth (Difco TSB) supplemented with yeast extract and glucose (2%). Bacteria were collected by centrifugation in the late logarithmic phase of growth and the supernatant was discarded. The bacteria were then washed with 0.1 M NaCl and, after the second

centrifugation, the pellet was suspended in 0.1 M NaCl and disrupted by sonic treatment. Centrifugation at 40,000 × g removed cell debris. The supernatant was collected and placed on the chromatography column. Each step of the preparation of the crude extract was done at 4 C.

Enzyme purification. The purified beta-lactamase was obtained from the crude extract by affinity chromatography on a cephalosporin C-bound column. The resin was prepared by coupling this antibiotic on Indubiose A4 following the cyanogen bromide precedure (1). The enzyme was eluted with an aqueous ampicillin solution (1 mg/ml) and concentrated by ultrafiltration through a Diaflo UM 10 membrane.

Production of anti-TEM serum. Anti-TEM antiserum was obtained from rabbits (New Zealand strain) who received the purified enzyme (TEM) intramuscular mixed with Freund complete adjuvant.

The animal was injected weekly over a 4-week period. Each injection consisted of 1 ml of TEM (10 U) mixed with 1 ml of adjuvant. The TEM unit refers to the enzyme concentration as follows: 0.3 ml of a solution containing 1 U induced an initial variation of 0.4 pH units per min when added to 9.7 ml of a NaCl solution (5 g/liter) containing 1 mg of ampicillin. The experiment was run at 38 C and pH 7.0.

The rabbit was bled 20 days after the last injection and the serum obtained was kept at −20 C.

Immunoelectrophoresis. The immunoelectrophoretic analysis was done on agarose gel under the procedure recommended by Pitton (15). Prior to analysis, the anti-TEM antiserum was adsorbed with a sensitive E. coli K-12 strain obtained from Pasteur Institut.

pH stat method. The beta-lactamase enzymatic activity was titrated with a Mettler pH stat (DK serial) following the procedure described by Labia et al. (7). The curve illustrating the volume of NaOH added to compense the pH variation as a function of time was recorded.

Measurements were done either with the enzyme alone or with the enzyme previously incubated with antiserum at room temperature. In the latter case, a pH stat typical experiment was run as follows: the cell, thermostatically controlled to 37 C, was filled with 9.6 ml of an NaCl solution (5 g/liter) containing 1 mg of ampicillin. pH was adjusted to 7.00. The serum-enzyme (1 U) mixture adjusted to pH 7.00 was added under a 0.4-ml volume. The titration was performed with a 5 × 10⁻³ M aqueous sodium hydroxide solution.

pH meter method. pH metric determination was run in the same conditions as those previously described for the pH stat. Starting the experiment at pH 7.00, the lowering of the pH in the cell was monitored and recorded in function of time until the enzyme reaction had ended. We used the Mettler pH meter (DK-10, DK-13 serial) connected to the Mettler GA 10 recorder.

RESULTS

Detection of anti-beta-lactamase antibodies with the pH stat. The initial velocity

of the enzymatic reaction was highly modified when the enzyme came into contact with the antibody. As shown in Table 1, the initial velocity value was unchanged with normal serum but was lowered from 100 to 8.5 when the enzyme and the antiserum were incubated for 40 min.

The neutralizing effect of anti-TEM antibodies was clearly demonstrated with the pH stat microacidimetric method.

The standard curve obtained when increasing quantities of antiserum were added to a fixed quantity of antigen is shown in Fig. 1.

When the enzyme was mixed with antiserum, the incubation time was an important parameter and greatly modified the degree of inhibition of the enzyme activity, as illustrated in Fig. 2.

Finally, it should be pointed out that, as the quantity of the antiserum increased, the kinetic of the reaction followed less and less the Mi-

TABLE 1. *Comparison of the initial velocity values with TEM alone or in combination*[a]

Conditions	Initial velocity (relative)
TEM + ampicillin	100
TEM + ampicillin + normal serum	100
TEM + ampicillin + immune serum	8.5

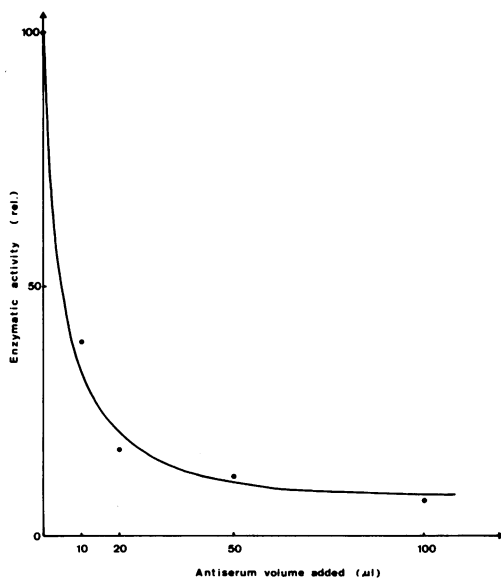[a] TEM was incubated 40 min with 0.1 ml of normal or immune serum.

FIG. 1. *Neutralization of TEM activity by anti-TEM antiserum. In the ordinate, the given values of enzymatic activity are the initial velocity values.*

chaelis-Menten model. When the curves were computerized, the correlation coefficient became worse.

Detection of antibodies with the pH meter. Figure 3 shows the different pH metric recordings for the enzyme activity.

As shown by the difference in total height of curves 1 and 2, there was an important buffer effect with 100 μliters of rabbit serum. However, curves 2 and 3 were clearly distinguishable,
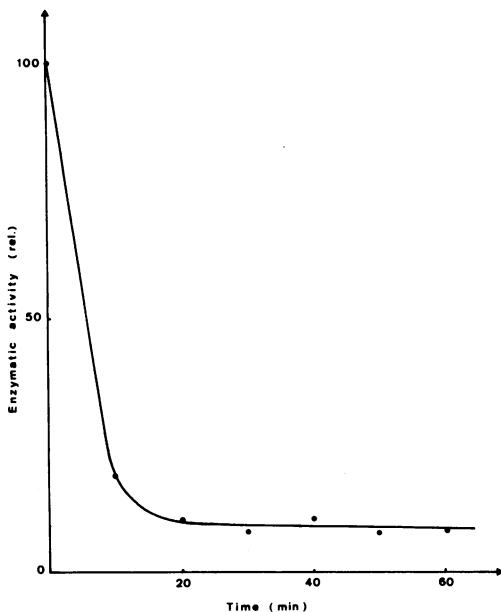

FIG. 2. *Effect of incubation time of serum with enzyme on the TEM activity neutralization. Enzyme (1 U) is incubated with 0.1 ml of antiserum at room temperature. In the ordinate, the given values of enzymatic activity are the initial velocity values.*
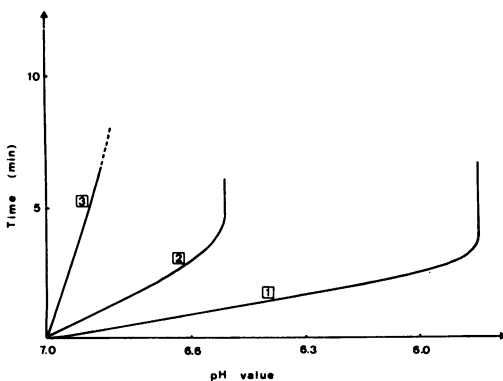

FIG. 3. *pH metric recordings of TEM activity. 1, TEM alone; 2, TEM with normal serum (0.1 ml); and 3, TEM with 0.1 ml of antiserum (due to antiserum inhibition, the velocity of the reaction is greatly reduced so that only the beginning of the reaction is recorded).*

particularly by their slope to the origin.

If we correct serum buffer effect, taking into account the difference in the height of the curves, we see that normal serum had a negligeable effect on enzymatic activity.

The percentage of the total pH variation obtained during the first minute of the reaction is shown in Table 2.

Specificity of the identification of the TEM beta-lactamase by the antiserum. The anti-TEM antiserum was tested against the P 453 beta-lactamase and the Marmot strain of *P. aeruginosa*. The results are summarized in the Table 3.

As seen by the pH stat method, there was no cross-reaction and the activity of the P 453 was unchanged in the presence of anti-TEM antiserum. When the crude Marmot extract was incubated with anti-TEM antiserum, there was a complete inhibition (90%) with ampicillin which is the TEM substrate, but there was no change of activity with cephalotin which is the cephalosporinase-like beta-lactamase.

Immunoelectrophoresis. As seen in Fig. 4, the immunoelectrophoresis of the anti-TEM antiserum gave one band of precipitation with the TEM purified enzyme. The antigen had migrated slightly towards the negative pole.

DISCUSSION

The microacidimetric pH stat method clearly illustrates the neutralizing capacity of anti-beta-lactamase antibodies. The antiserum modifies the initial slope of the curve obtained when the volume of sodium hydroxide added is plotted in function of the time. This slope is proportional to the initial velocity of the enzymatic reaction and can be compared to the V_{max}, because, at that degree of the reaction, there is an excess of substrate. Moreover, the curve is linear at its beginning. Measured with the pH stat, the maximal degree of neutralization of the TEM enzyme is over 90%.

The effect on the initial velocity can be recorded by use of a simple pH meter. Though the results are not as distinct as those obtained with the pH stat, due to buffer properties of the rabbit serum, the pH meter method is suitable for a rapid identification of a beta-lactamase, and the results obtained are highly significant.

By means of microacidimetric methods and antiserum, the TEM beta-lactamase has been easily detected and identified from crude bacterial extracts. This should be the same for any wild-type bacterial strain carrying the TEM plasmid. It is now possible to rapidly detect the presence of a beta-lactamase without the crucial step of enzyme purification and substrate study.

TABLE 2. *Comparison of the percentage of the total pH variation during the first minute of the reaction with TEM alone or in combination*[a]

Conditions	$\Delta pH/pH_{tot}$ (%)
TEM + ampicillin	34
TEM + ampicillin + normal serum	30
TEM + ampicillin + immune serum	5

[a] TEM was incubated 40 min with 0.1 ml of normal or immune serum.

TABLE 3. *Neutralizing action of anti-TEM antiserum on various beta-lactamases*

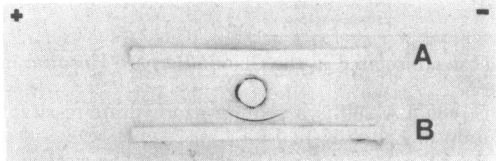| Enzyme	Substrate	Inhibition (%)
TEM	Ampicillin	91
P453	Ampicillin	None
Marmot	Ampicillin	90
	Cephalotin	None

FIG. 4. *Immunoelectrophoretic analysis. A, Normal serum; B, anti-TEM antiserum. Center well: TEM purified.*

The accuracy of the acidimetric methods is very high, because it is the enzymatic reaction itself that is directly measured without perturbations due to the experimental conditions. It is an advantage over the iodometric method, in which there are significant inconveniences due to interaction either with the enzyme or the antiserum.

The acidimetric methods seem to be of higher specificity than iodometric methods. They easily differentiate two beta-lactamases which are kinetically very close, the R-TEM and the P 453 (16). Roupas and Pitton (15) has shown that these two enzymes belong to different immunotypes (type I and II, as a result of immunoelectrophoresis). These enzymes have not been differentiated by the iodometric method.

The results obtained with the P 453 and Marmot bacterial extracts prove that the microacidimetric enzyme neutralization test is a powerful tool for the detection of the TEM beta-lactamase. The experimentation on the *P. aeruginosa* has shown that even in a mixture of beta-lactamases, the TEM is easily identified.

The immunological procedures described here could be seen at large. One can expect the production of antisera to other classes of well-defined beta-lactamases to be implicated frequently in the bacterial resistance to antibiotics. Immunoacidimetry could be useful to gain precision in the classification of these enzymes and to obtain epidemiological data on the dispersion and the frequency of the beta-lactamases.

ACKNOWLEDGMENTS

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