IMMUNOLOGICAL STUDIES OF AMINOGLYCOSIDE 3′-PHOSPHOTRANSFERASES

Sir:

As previously reported[1], aminoglycoside phosphotransferase II which transfers the terminal phosphate of ATP to the 3′-hydroxyl group of the 6-amino-6-deoxy-D-glucose or 2,6-diamino-2,6-dideoxy-D-glucose moiety of kanamycins A and B and butirosins, but not to the 5-hydroxyl group of the D-ribose moiety of lividomycins has been completely purified from *Escherichia coli* JR66/W677. The enzyme (100 μg as protein) thus purified separated into two bands during polyacrylamide gel electrophoresis 7.5, 10 or 15% at pH 8.9; the bands were detected by the method of HARTMAN and UDENFRIEND using 8-anilino-1-naphthalene sulfonate[2]. The two bands were sliced from the 10% gel, and homogenized with 2.0 ml of 0.9% sodium chloride. The protein content of the fast-moving band was 84 μg and that of the slowly moving band was 16 μg. Both of them showed the same activity in inactivating butirosins and kanamycin A. The inactivation (enzymatic activity) was tested as described in a previous paper[3] by determining the residual antibiotic; in case of butirosins, 100% inactivation was produced by the fast-moving fraction and 60% by the slowly moving fraction; in case of kanamycin A, 100% inactivation was produced by the fast-moving fraction and 30% by the slowly moving fraction. The slowly moving fraction appeared after treatment (lyophilization, Sephadex G-25 chromatography and lyophilization successively) of the fast-moving fraction and it was suggested that the enzyme in the slowly moving band might be produced from the fast-moving fraction. Both were used for preparation of their rabbit antisera.

Each of above homogenates containing 84 μg of protein in the fast-moving band or 16 μg of protein in the slowly moving band was mixed with an equal volume of complete FREUND's adjuvant (Difco Laboratories, Inc) and 2.0 ml of the mixture were inoculated subcutaneously in four parts on the backs of two rabbits (each 2.5 kg, male). The materials for immunization were prepared just before their use, by gel electrophoresis as described above. The inoculation was repeated three times at 2-week intervals. Forty-three days after the initial inoculation, a homogenate of each band in 0.9% sodium chloride solution was inoculated without adjuvant. The rabbits were killed and bled 7 days after the last inoculation and the antisera were treated by heating at 55°C for 30 minutes. The presence of the antibody was shown by inhibition of APH (3′)-II activity, immunodiffusion[4] and immunoelectrophoresis. The antisera from two rabbits immunized with the homogenate of the fast-moving band were diluted 64 times and the diluted sera (0.1 ml) was mixed with 0.1 ml of the enzyme solution (1 μg as protein/ml) for 20 minutes at 37°C and the inactivation of butirosins was determined by the method described in the previous paper[3]. One of the preparations showed 100% inhibition and the other 65% inhibition of enzymatic activity respectively; the antisera of two rabbits immunized by the homogenate of the slow-moving band were tested similarly and one showed 80% inhibition and the other 20% inhibition. The antisera obtained against the enzymes of the two bands showed complete cross reaction and neither serum inhibited the activity of APH(3′)-I. Therefore, the antiserum to the fast-moving band described above was studied in detail as described below, against aminoglycoside phosphotransferases isolated from a variety of resistant strains.

Each resistant strain of bacteria was grown at 37°C for 16 hours in a medium (pH 7.4) containing 1% peptone, 0.5% meat extract and 0.3% sodium chloride. The cells were harvested by centrifugation, washed twice with buffer A which consisted of 20 mM phosphate buffer (pH 7.2), 10 mM magnesium acetate, 60 mM potassium chloride and 2 mM 1,4-dithiothreitol and suspended in an equal volume of buffer A. The cell suspension was passed through a French pressure cell under a pressure of 1,200 kg/cm^2 and the disrupted-cell suspension was centrifuged at 10,000 *g* for 60 minutes. The supernatant thus obtained was called the crude enzyme. Using this crude enzyme, the reaction with the antibody was tested by immunodiffusion (Fig. 1a and b).

As shown in Fig. 1a, a precipitin line was observed between the antisera in the center well and the purified or the crude APH(3′)-II. The antisera was specific to the APH(3′)-II and did not produce a precipitin line with the other crude enzymes such as those of APH(3′)-I prepared from *E. coli* K-12 J5 R11-2 or from *P. aeruginosa* TI-13. As shown in Fig. 1b, the crude enzymes of

APH(3′)-II prepared from *E. coli* JR66/W677 and from *P. aeruginosa* H9 both showed a single continuous precipitin line with the antisera; the crude enzyme of APH(3′)-III prepared from *P. aeruginosa* 21–75 did not form a precipitin line. As previously reported[3], *P. aeruginosa* B-13 forms APH(3′)-I and a trace amount of APH (3′)-II. The crude enzyme prepared from this strain showed no appreciable amount of APH (3′)-II, that is, no activity to inactivate butirosins and did not produce a precipitin line with the APH(3′)-II antisera. The APH(3′)-II antiserum inhibited the APH(3′)-II activity contained in the crude enzymes prepared from *P. aeruginosa* H9. In contrast, the activities of APH(3′)-I in the crude enzymes prepared from *E. coli* K-12 J5 R11-2, from *P. aeruginosa* TI-13 and from *P. aeruginosa* B-13 were not inhibited by the APH (3′)-II antiserum. Therefore, we have confirmed that the APH(3′)-II enzymes obtained from *E. coli* and *P. aeruginosa* are identical both in immunological specificity and in substrate specificity. As reported in a previous paper[3], APH (3′)-II enzymes obtained from these strains have the same characteristics of chromatographic behavior, molecular weight, optimal pH and Km value. These results suggest that the APH(3′)-II enzymes in these resistant bacteria may be derived from the same origin. As described above APH(3′)-II can be clearly differentiated from APH(3′)-I and APH(3′)-III by immunological specificity.

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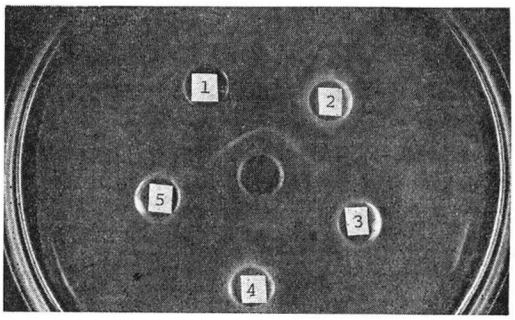
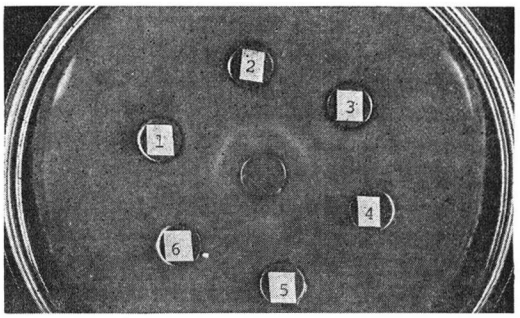
Fig. 1. Immunodiffusion patterns of an antiserum to APH(3′)-II.



a. The center well contained the antisera to APH (3′)-II of *E. coli* JR66/W677. Well 1 contained the purified APH(3′)-II of *E. coli* JR66/W677. Wells 2 and 4 contained the crude enzyme of *E. coli* JR66/W677. Well 3 contained the crude enzyme of *E. coli* K-12 J5 R11-2. Well 5 contained the crude enzyme of *P. aeruginosa* TI-13.



b. The center well contained the antisera to APH (3′)-II of *E. coli* JR66/W677. Wells 1, 3 and 5 contained the crude enzyme of *E. coli* JR66/W677. Well 2 contained the crude enzyme of *P. aeruginosa* H9. Well 4 contained the crude enzyme of *P. aeruginosa* 21–75. Well 6 contained the crude enzyme of *P. aeruginosa* B-13.

References

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