CLASSIFICATION OF *PSEUDOMONAS AERUGINOSA* O ANTIGENS BY IMMUNOELECTROPHORESIS

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IN a study reported previously (Lányi and Ádám, 1973), it was shown that double agar-gel precipitation and agglutination give, as a rule, identical cross-reaction patterns for the O antigens of *Pseudomonas aeruginosa*. On the basis of precipitation of different kinds of bacterial extract, the antigens were classified into the following groups: (1) those of which saline extracts, supernates of phenol-water extracts (L_1 fractions) and purified lipopolysaccharide (LPS) precipitated readily with homologous antisera; (2) those in which saline extracts and LPS precipitated but L_1 fractions did not; and (3) two partial antigens, none of the extracts of which precipitated with absorbed antisera, although the presence of the antigen was demonstrable by means of agglutination tests. In the experiments described in this paper, a further classification of *P. aeruginosa* O antigens was attempted.

MATERIALS AND METHODS

Bacterial strains. Twenty-three *P. aeruginosa* cultures represented Lányi's O-antigen type strains (Lányi, 1966-67 and 1970); in addition, 53 strains of different serogroups were chosen from our collection. Of 13 strains received from Professor P. V. Liu, Louisville, Kentucky, USA, as type strains of the provisional international *P. aeruginosa* antigenic scheme, 12 originated from Habs (1957) and one from Sandvik (1960). Saline extracts were made from all these cultures, but only Lányi's 23 O-antigen type strains were used for the preparation of L_1 , LPS, trichloroacetic-acid (TCA) and alkali extracts.

Antigens. Extracts in physiological saline ("saline") were prepared by heating the bacterial suspension at 100°C for 2½ h; each ml of the supernate obtained after centrifugation represented c. 30 mg of bacteria (moist weight). L_1 fractions were the freeze-dried supernates of ultracentrifuged phenol-water extracts; nucleic acid-free LPS preparations were dialysed and freeze-dried phenol-water extracts purified by ultracentrifugation at 105,000 g. L_1 and LPS solutions were prepared for precipitation by dissolving 5 mg of the freeze-dried preparation in saline and heating at 100°C for 1 h. The preparation of these antigens was described in detail by Ádám, Kontrohr and Horváth (1971) and by Lányi and Ádám (1973).

The bacteria were, as a rule, grown in Roux flasks at 37°C for 24 h on nutrient-agar medium: beef extract (Central Slaughterhouse, Budapest) 15 g; peptone (Richter, Budapest) 10 g; NaCl 3 g; Na₂HPO₄ · 12H₂O 4 g; agar 18 g; tap water 1000 ml; pH 7·4. Blood-agar cultures were grown on plates containing baker's yeast 40 g; peptone (Richter, Budapest) 10 g; NaCl 3 g; Na₂HPO₄ · 12H₂O 4 g; agar 18 g; tap water 1000 ml; pH 7·4; ox blood 50 ml.

TCA extracts were prepared by suspending 5 g of dry bacteria or 10 g of moist bacteria in 50 ml of ice-cold TCA 10% (w/v). The suspension was left to stand in the refrigerator at 4°-6°C for 24-48 h and then centrifuged. After a second extraction of the deposit, the

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supernates were pooled and dialysed. The concentrated and freeze-dried extract was dissolved in saline (2 mg per ml) and used without heating.

Alkali extracts were made by suspending washed bacteria in 0.5M sodium hydroxide and heating at 80°C. At intervals, samples were removed from the water bath, neutralised with hydrochloric acid and used as antigens for immunoelectrophoresis. The salts were removed by dialysis from several of the alkali-treated extracts. Each ml of alkali extract represented c. 30 mg of bacteria (moist weight).

Antisera were prepared in rabbits with bacteria heated at 75°C for 1 h (Lányi, 1966-67).

Immunoelectrophoresis. Each slide (25 × 75 mm) was covered with 3.5 ml of gel containing K_2HPO_4 , 2.61 g; KH_2PO_4 , 2.04 g; Oxoid Agar No. 1, 9 g; merthiolate, 0.1 g; water, 1000 ml. The extracts were electrophoresed in 10- μ l amounts from wells 3 mm in diameter with merthiolate-treated potassium-phosphate buffer (0.03M, pH 6.9) at a gradient of 6 V per cm and at 3.7 mA per slide for 120 min. At the end of electrophoresis, a median

FIG. 1.—Precipitation arcs obtained with saline extracts of *Pseudomonas aeruginosa* antigens. a = immunoelectrophoretic (IE)-groups Ia and Ib; b = IE-group Ic; c = IE-group II; d = IE-group III; e = IE-group IV; f = IE-group V. Anode on left; each division on the scale = 10 mm.

trench, 1.5 mm in width was cut parallel to the line of current flow and filled with 50 μ l of antiserum. Then the slides were incubated in a moist chamber at 37°C and examined after incubation for 24 h. Staining was performed with amido-black (Uriel, 1971).

Recording of the position and intensity of the precipitation arcs. Stained and dried slides were recorded in the Zeiss ERI 65 m automatic densitometer supplied with a 560-nm filter. Photometric densities of the arcs were plotted against the distance along the long axis of the slide on both the anode (left-hand) and the cathode (right-hand) side of the well.

Designation of precipitation lines. The arcs were arbitrarily termed, according to localisation, A⁺ (+30 to +15 mm), A (+20 to +8 mm), B (+10 to 0 mm), C (+10 to -5 mm), D (0 to -20 mm). Double or triple lines were termed A₁, A₂ etc. Precipitates probably not identical with lines A, B, C or D, but showing the same migration rate, were designated respectively A_x, B_x, C_x and D_x.

RESULTS

Immunoelectrophoretic patterns of saline extracts

Saline extracts of a total of 89 strains representing all recognised *P. aeruginosa* antigens were electrophoresed against the homologous antisera. The cultures fell into distinct immunoelectrophoretic (IE) groups, some of which, on the basis of the behaviour of other kinds of extract, were classified into IE subgroups. As seen in fig. 1, members of IE-groups Ia and Ib were characterised by a thick arc toward the positive electrode (A) and a somewhat thinner line (B) that started from the edge of the antigen well and usually fused at its anodic end with arc A. Subgroup Ic showed lines somewhat different from this pattern. Cultures classified into IE-group II gave a line (C₁) ranging from 10-15 mm on the positive to 10-15 mm on the negative side of the antigen well, one shorter line (C₂) more towards the cathode side, and a short line (B_x) extending from the starting well towards the anode. IE-group III gave lines C₁ and C₂, and a short line (D_x) towards the negative electrode. IE-group IV

TABLE

Immunoelectrophoretic (IE) characteristics of Pseudomonas aeruginosa antigens

IE group	Precipitation arcs, in tests with homologous antiserum, given by						O antigens*
	saline extract	L ₁ fraction	LPS fraction	TCA extract	alkali extract		
					(NaOH 1 h)	(NaOH 24 h)	
Ia	A, B	A, B	A, B, C _x	A, B, C _x	A ⁺	—	O2 (Habs 10); O3a, 3b; O3c (Habs 2); O3a, 3d (Habs 5); O3a, 3d, 3e; O3d, 3f
Ib	A, B	A, B	A, B, C _x	A, B, C _x	A	—	O1 (Habs 3); O6 (Habs 1)
Ic	A, B	A, B	A, B, C _x	A, B, C _x	—	—	O4a, 4c; O5a, 5b, 5c; O5a, 5b, 5d (Habs 8); O5a, 5d; O10a (Habs 9); O10a, 10b
II	B _x , C ₁ , C ₂	C ₁ , C ₂	C ₁ , C ₂ , C _x	C ₁ , C ₂ , C _x	—	—	O4a, 4b; O4a, 4d
IIIa	C ₁ , C ₂ , D _x	C ₁ , C ₂	C ₁ , C ₂ , C _x	C ₁ , C ₂ , C _x	C	C	O12
IIIb	C ₁ , C ₂ , D _x	—	C _x	C ₁ , C ₂ , C _x	A	A	O13 (Habs 12)
IV	D ₁ , D ₂ , D _x	—	C _x	D ₁ , D ₂ , C _x	D	D	O7a, 7b (Habs 11); O7a, 7c; O11 (Habs 4)
V	A _x	—	A _x , C _x	—	—	—	O8; O9

* O antigens are designated according to Lányi's antigenic scheme (1966-67); bracketed figures indicate the corresponding groups of Habs (1957).

— = No arc seen.

Fig. 2.—Densitometric curves representing the main immunoelectrophoretic types obtained with saline extracts of *P. aeruginosa*: — = IE-group Ia (strain O3a, 3d); = IE-group IIIa (strain O12); - - - - = IE-group IV (strain O7a, 7b); - · - · = IE-group V (strain O8).

strains formed, in addition to line D_x , two arcs on the cathode side (D_1 and D_2). Two strains, no. 170017 (O-group 8) and no. 170018 (O-group 9), which were placed in a separate IE-group V, differed from all other cultures in exhibiting only a thin line at the site of line A (A_x). A visible line A_x was produced only if the extract had been prepared from 3-day-old blood-agar cultures containing about three times the usual amount of bacteria (see *Materials and methods*). Precipitation arcs obtained with saline extracts are listed in the 2nd column of the table.

For a more accurate comparison of density and sites of the precipitation arcs, stained immunoelectrophoretic slides were recorded by microdensitometry. Fig. 2 shows that lines distinguishing the IE groups can be well demonstrated by photoelectric measurement. Arc A contained the greatest amount of precipitate; the other line characteristic of IE-group I was next in order. Line A ranged from +20 to +8 mm and had a peak at +15 mm, while line B ranged from +10 to 0 mm with a peak at +8 mm, where it fused with line A. IE-group II (not shown in fig. 2) had a density curve between +10 and -10 mm, with a peak at 0 mm. IE-group-III antigens were characterised by curves shifted more to the negative side (+3 to -15 mm), with a peak at -5 mm. IE-group-IV extracts precipitated definitely towards the cathode, ranging from about 0 to -20 mm, but formed a peak practically at the same site as group-III antigens. The usually multiple arcs for strains of IE-groups II, III and IV consisted frequently of faint lines; the relatively high peaks are explained by the fact that photoelectric measurement reflected the total density of precipitates falling at the same site along the long axis of the slide. The low peak of line A_x is in agreement with the faint precipitation shown by IE-group-V strains. Density curves were localised similarly for all strains belonging to the same IE group, only the amplitude of the curve varied with the amount of precipitate formed, that is, with the titre of the serum.

FIG. 3.—Precipitation arcs obtained with *P. aeruginosa* L₁ and LPS fractions: IE-group I, *a* = L₁ and *b* = LPS; IE-groups II and IIIa, *c* = L₁ and *d* = LPS; IE-groups IIIb and IV, *e* = L₁ and *f* = LPS; IE-group V, *g* = L₁ and *h* = LPS. See also legend to fig. 1.

Immunoelectrophoretic patterns of L₁ and LPS extracts

The immunoelectrophoretic patterns given by L₁ and LPS preparations of the 23 type strains of Lányi's antigenic scheme are shown in fig. 3. With the majority of strains, the L₁ extracts gave patterns very similar to those of the saline extracts; but with IE-group-I strains, the L₁ extracts—unlike saline extracts—usually formed a double B line (B₁, B₂) and arc A also frequently consisted of two parallel lines (A₁, A₂). The L₁ fractions of strains of IE-groups IIIb, IV and V failed to precipitate (3rd column of the table).

LPS fractions of IE group-I, -II and -IIIa strains gave similar results to those obtained with the corresponding saline extracts, except that a short arc at

FIG. 4.—Densitometric curves representing the main immunoelectrophoretic types obtained with LPS fractions of *P. aeruginosa*; — = IE-group Ia (strain O3a, 3d); = IE-group II (strain O4a, 4d); - - - - = IE-group IV (strain O7a, 7b).

FIG. 5.—Precipitation arcs obtained with *P. aeruginosa* antigens after exposure to 0.5N sodium hydroxide for 1 h at 80°C: a = IE-group Ia; b = IE-group Ib; c = IE-group IIIa; d = IE-group IV. See also legend to fig. 1.

FIG. 6.—Densitometric curves for precipitation arcs obtained with *P. aeruginosa* antigens after exposure to 0.5M sodium hydroxide for 1 h at 80°C: --- = IE-group Ia (strain O3a, 3d); — = IE-group Ib (strain O6); = IE-group IIIa (strain O12); - · - · - = IE-group IV (strain O7a, 7b).

the site of the starting well (C_x) was always present; traces of this arc were sometimes observed with L_1 fractions. LPS fractions of strains of groups IIIb and IV differed from the rest in the absence of lines corresponding to those produced with saline extracts; arc C_x , however, was formed by these antigens (4th column of the table).

Fig. 4 shows densitometric recordings for some LPS fractions. The similarity of the LPS curves to the saline-extract curves (fig. 2) is evident for IE-groups I and II. The definite peak at 0 mm in the LPS curves is due to the presence of line C_x . IE-group IV, the LPS fraction of which formed only arc C_x , exhibited a sharply rising and falling curve at the site of the antigen well.

Immunoelectrophoretic patterns of trichloroacetic acid extracts

As shown in the 5th column of the table, the TCA extracts usually gave precipitation arcs similar to those of the corresponding LPS fractions, that is, they showed the presence of a short arc at the starting well (C_x) in addition to lines seen with saline extracts. TCA extracts of strains of IE-group IV differed from LPS fractions in the presence of lines D_1 and D_2 . TCA extracts of group-V strains failed to react.

Immunoelectrophoretic patterns of alkali extracts

Heating at 80°C with 0.5M sodium hydroxide allowed a further subdivision of *P. aeruginosa* antigens; fig. 5 shows the main types of precipitation arcs and fig. 6 demonstrates the main types of density curves observed. After treatment for 1 h with alkali, antigens of IE-group Ia precipitated more toward the anode than did those in the corresponding saline extracts and thus formed a new arc termed A^+ ; alkali-treated antigens of IE-group Ib showed the

usual migration rate (5th column of the table). These extracts were moderately resistant to alkali in that they still reacted after exposure for 5 h but not after 24 h. Antigens belonging to IE-groups III and IV were highly resistant to alkali. It is remarkable that the corresponding saline extracts in groups III and IV gave multiple precipitation lines, whereas the alkali-treated antigens usually formed single arcs. The rest of the antigens, belonging to IE-groups Ic, II and IV, were sensitive to alkali, losing their precipitating ability after exposure to sodium hydroxide for 15–60 min.

Classification of *P. aeruginosa* antigens, determined on the basis of agglutination, into immunoelectrophoretic groups is summarised in the table.

Cross-reactions between related antigenic groups

Extracts of type strains shown by agglutination (Lányi, 1966-67) and by double agar-gel precipitation (Lányi and Ádám, 1973) to share major partial antigens gave the corresponding cross-reactions in immunoelectrophoresis. The lines developing with the heterologous antiserum were usually less marked than those with the homologous serum, but appeared always at the same site and in a closely similar form.

DISCUSSION

The results of the present experiments showed a close correspondence between the electrophoretic mobility and the serological classification of *P. aeruginosa* antigens; strains falling into the same antigenic group or sharing major partial antigens exhibited the same immunoelectrophoretic pattern. Strains with the O antigen 4a, 4c differed in this respect from other O4 cultures, but in the rest of the properties examined (alkali sensitivity, behaviour of L₁ and LPS fractions) all O4 strains were uniform.

The failure of L₁ fractions of antigens O7a, 7b, O7a, 7c, O8, O9, O11 and O13 to precipitate was first observed by Ádám and Kontrohr (1970, unpublished data) and was described by Lányi and Ádám (1973). Because lines A and B, C₁ and C₂, as well as D₁ and D₂, represent probably the side chain of the antigen molecule, it may be assumed that the above antigens contained no side chains appearing in the L₁ fraction.

The unusual behaviour of antigens O8 and O9 deserves some interest. These two cultures differed from the others in that they produced weakly reacting saline and LPS extracts, their TCA fractions failed to precipitate and their suspensions were only moderately immunogenic in rabbits. These findings confirm earlier assumptions (Ádám *et al.*, 1971; Lányi and Ádám, 1973) that the main antigens in these strains are R-like factors. It is noticeable that line C_x, a short, usually semicircular arc at the starting well, was present in the LPS fraction of all strains, including O8 and O9. Accordingly, arc C_x may represent the core of the antigen molecule. The finding that line A_x appeared only with the saline and LPS extracts of strains O8 and O9, also indicates the unusual structure of these antigens.

The sensitivity to alkali treatment of certain antigens may be associated with the O-acetyl group content of their side chains.

The present experiments are in agreement with the finding of van Eeden (1967) that trichloroacetic acid extracts of *P. aeruginosa* strains exhibited different immunoelectrophoretic patterns. Antigens of van Eeden's four strains running towards the anode correspond to our IE-group I; antigens precipitating on the cathode side are comparable with our group-IV antigens, while those appearing on both sides of the antigen well may belong to our groups II and III.

SUMMARY

Heated saline extracts of 89 strains, and (1) supernates of phenol-water extracts (L_1 fractions), (2) purified lipopolysaccharide, (3) trichloroacetic-acid (TCA) extracts, and (4) sodium-hydroxide extracts of 23 strains representing all *Pseudomonas aeruginosa* O antigens were subjected to electrophoresis. Precipitation lines obtained with homologous and heterologous antisera were evaluated by electrodensitometric measurement. The characteristics of the immunoelectrophoretic groups established were as follows. *Group I*: two lines running at different rates towards the anode; three subgroups on the basis of the behaviour of alkali-treated antigens. *Group II*: triple line at the starting well, alkali sensitive. *Group III*: triple line at the starting well, alkali resistant; two subgroups according to reactivity or non-reactivity of L_1 fractions. *Group IV*: triple line on the cathode side, alkali resistant, L_1 fraction non-reactive. *Group V*: single line on the anode side, alkali sensitive, L_1 fraction and TCA extract non-reactive. O antigens identified by agglutination corresponded closely with the immunoelectrophoretic pattern: strains with identical O antigens or sharing major somatic components fell, with one exception, into the same immunoelectrophoretic group.

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