GEL DIFFUSION TECHNIQUE IN ANTIGEN-ANTIBODY REACTIONS OF *ACTINOMYCES* SPECIES AND "ANAEROBIC DIPHTHEROIDS"

SYLVIA KING AND ESTHER MEYER

Hektoen Institute for Medical Research of the Cook County Hospital, and Department of Microbiology, College of Medicine, University of Illinois, Chicago, Illinois

Received for publication 13 August 1962

ABSTRACT

KING, SYLVIA (Hektoen Institute, Chicago, Ill.) AND ESTHER MEYER. Gel diffusion technique in antigen-antibody reactions of *Actinomyces* species and "anaerobic diphtheroids." J. Bacteriol. **85**:186-190. 1963.—The Ouchterlony agar gel diffusion test was used to establish antigenic patterns produced by *Actinomyces israelii*, *A. bovis*, and *A. naeslundii*, as compared with those of "anaerobic diphtheroids." The organisms studied included *Actinomyces* species isolated from cases of human and bovine actinomycosis, and from the normal oral cavity of human volunteers. The "anaerobic diphtheroids" were obtained from a variety of clinical conditions. *A. israelii* was serologically distinct from *A. bovis*, and *A. naeslundii* shared a minor component with the other two *Actinomyces* species. There were no cross reactions between the "anaerobic diphtheroids" and any of the *Actinomyces* species.

To our knowledge, the agar gel diffusion technique of Ouchterlony (1949) has not been used in studies of *Actinomyces* species. However, some investigators have employed this method with fungi belonging to the *Eumycetes* group: Kaden (1956), *Sporotrichum schenkii*; Seeliger (1956), *Madurella grisea*; Heiner (1958), *Histoplasma capsulatum*; and others.

Using cultural and biochemical methods, Thompson and Lovestedt (1951) studied 11 oral isolates, and found that 2 were identical to *A. israelii* and 9 were another species of *Actinomyces* which grew under both aerobic and anaerobic conditions. They proposed the name *A. naeslundii*, since Naeslund (1925) originally described such a facultative isolate. Howell et al. (1959) compared 211 strains of *Actinomyces* isolated from the oral cavity, and found these strains were of two types, *A. israelii* and *A. naeslundii*. Suter (1956) reported the catalase reaction to be a good screening test for separating *Actinomyces*, which are catalase-negative, from the catalase-positive *Corynebacterium* species. King and Meyer (1957) confirmed these findings and suggested three differential carbohydrates useful in separating *A. israelii* and *A. bovis* from the "anaerobic diphtheroids."

Contradictory results have been published based on agglutination, complement fixation, and precipitin tests. Goyal (1938), using the complement-fixation test with methyl alcohol-extracted antigens, observed cross reactions between *Actinomyces* species and diphtheroids. Lentze (1938), employing heat-killed antigens for the agglutination test, failed to demonstrate homogeneity within the *A. bovis* species.

Erikson (1940) showed no cross reactions between *A. israelii* and *A. bovis*, using the agglutination test. This finding was confirmed by King and Meyer (1957). Slack et al. (1955) studied microaerophilic *Actinomyces* isolated from human and animal sources, using the agglutinin adsorption technique. They found that these could be divided into groups irrespective of their original habitat.

Recent studies by Slack et al. (1961), using fluorescent antibody techniques, demonstrated cross reactivity between *A. israelii*, *A. bovis*, *A. naeslundii*, and "anaerobic diphtheroids."

This report is concerned with immunological interrelationships among *Actinomyces* species, as determined by double-diffusion agar analysis. Additionally, evaluation of relatedness between *Actinomyces* species and "anaerobic diphtheroids" is reported.

MATERIALS AND METHODS

The microorganisms used and their origins are listed in Table 1.

Antigen preparation. The organisms were grown in brain liver heart infusion broth, using

TABLE 1. *Microorganisms*

Isolated from	Source of culture	Genus and species
Human actinomycosis		
CC2, CC5, CC7, CC8, Hill	King and Meyer, Chicago	*Actinomyces israelii*
Hubble	Hubble, Ralph H., Chicago	*A. israelii*
50155, 53163, 5561	Hazen	*A. israelii* (*A. bovis*)*
12102	ATCC, Howell	*A. israelii*
8373, 10048	ATCC, Emmons	*A. israelii* (*A. bovis*)*
9947	Record unavailable	*A. israelii* (*A. bovis*)*
Bovine actinomycosis		
Holmgren	King and Meyer, Chicago	*A. israelii* (*A. bovis*)†
P1, P2S	Pine, Duke Univ.	*A. bovis*
C26	Cummins, London	*A. bovis*
Normal oral cavity		
NN1, NN2, NN3, Yale	King and Meyer, Chicago	*A. israelii*
Newsome, Neuhaus, White	King and Meyer, Chicago	*A. israelii*
Bloomer	King and Meyer, Chicago	*A. israelii*
Bibbs	King and Meyer, Chicago	*A. naeslundii*
CS838	Howell, NIH	*A. israelii*
CS768	Howell, NIH	*A. naeslundii*
12104	ATCC, Howell	*A. naeslundii*
Various clinical conditions		
D14, 55, 438, 1333, 01, 02, 175, 1377	King and Meyer, Chicago	*Corynebacterium* sp.
11482	ATCC, Hungate	*C. acnes*

* These organisms were identified as *A. bovis* prior to the time that the species *A. israelii* was officially accepted by the *Bergey Manual*, and should be named *A. israelii*.

† This organism was identified as *A. israelii* by Cummins (1962), Howell et al. (1959), and King and Meyer (1957).

Brain Liver Heart semisolid medium (Difco) which was dissolved in cold distilled water and the agar removed by filtration. This medium was dispensed in 250-ml quantities in 500-ml flasks, and was sterilized by autoclaving at 15 lb of pressure for 20 min. The flasks were seeded with approximately 1 ml of an actively growing culture, and were incubated anaerobically for 10 to 14 days at 37 C. The microorganisms were removed by centrifugation at 732 × *g* for 30 min. Cold acetone (2 volumes) was added to 1 volume of the clear supernatant fluid, and this mixture was refrigerated overnight. The resulting precipitate was dissolved in a minimal amount of distilled water. Each antigen was standardized to contain 0.4 mg of total nitrogen per ml.

Antisera preparation. Male albino rabbits (2 kg), previously trial bled, were immunized with cells which had been homogenized for 4 min in a Servall Omni-mixer (115 v, 16,000 rev/min, rheostat 60). The immunization schedule was that employed by Kroeger and Sibal (1961).

Agar gel diffusion technique. Noble Agar (Difco), 0.8%, in phosphate-buffered saline (pH 7.2) was autoclaved, cooled slightly, and Merthiolate was added to a final concentration of 1:10,000. [Equally satisfactory results were obtained using phenolized agar, as described by Schubert, Lynch, and Ajello (1961).] Then 8 ml of agar were poured into 60-mm petri dishes, the gel was allowed to harden, and the plates were refrigerated for at least 1 to 2 hr. Wells were cut out with a Feinberg 5-hole cutter (Shandon Scientific Co., Ltd., London), and were sealed with one

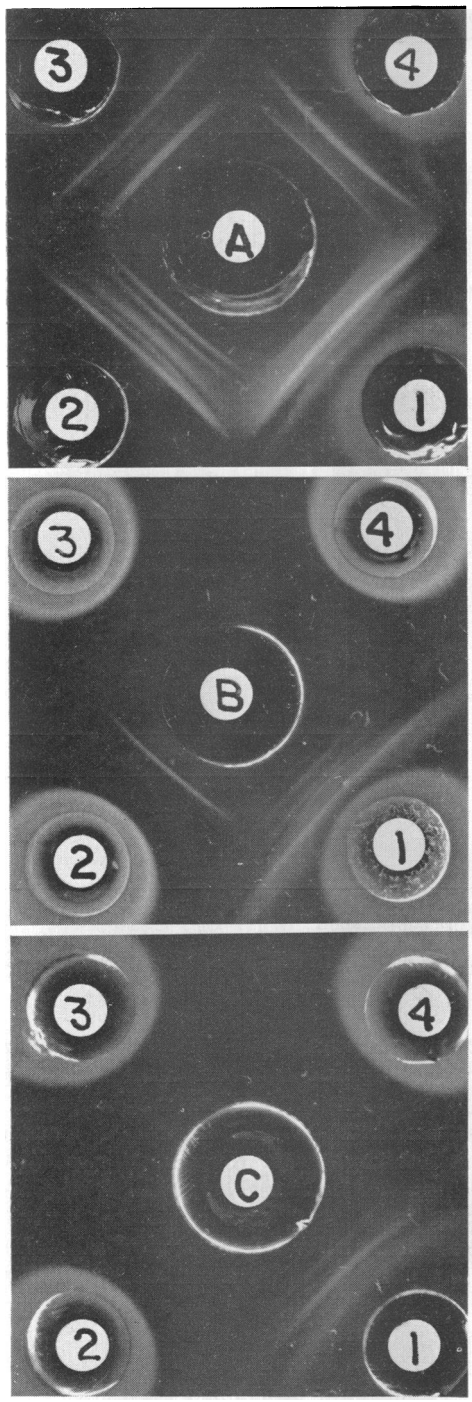

FIG. 1. *Double-diffusion precipitin reactions.* (A) *Actinomyces israelii (Holmgren) antigen* × (1) *A. israelii (Holmgren) antisera,* (2) *A. israelii (White) antisera,* (3) *A. israelii (Yale) antisera,* (4) *A. israelii (Newsome) antisera.* (B) *A. naeslundii antigen* × (1) *A. naeslundii antisera,* (2) *A. israelii (Hill) antisera,* (3) *diphtheroid (D438) antisera,* (4) *diphtheroid (D1333) antisera.* (C) *A. bovis (P1) antigen* × (1) *A. bovis (P1) antisera,* (2) *A. israelii (Hill) antisera,* (3) *diphtheroid (438) antisera,* (4) *diphtheroid (D1333) antisera.*

TABLE 2. *Agar gel precipitin tests, summary of results*

Antigen	Antisera			
	Actinomyces israelii	*A. bovis*	*A. naeslundii*	"Anaerobic diphtheroids"
A. israelii	+	−	−	−
*A. israelii (A. bovis)**	+	−	−	−
A. bovis	−	+	−	−
A. naeslundii	+	+	+	−
"Anaerobic diphtheroids"	−	−	−	V†

* See footnote to Table 1.
† Variable.

drop of molten agar. The center well was filled with antigen, and the peripheral wells were filled with the antisera. Precipitin bands were observed and photographed after the plates were incubated for 3 days at 37 C. All tests were repeated at least three to four times for verification.

RESULTS

All of the *A. israelii* antigens, when screened against heterologous *A. israelii* antisera, produced two to five precipitin bands. Antigens prepared from each of the isolates showed at least two precipitin bands in common with every other *A. israelii* antigen (Fig. 1A). All of the *A. israelii* antisera formed one line of precipitation with *A. naeslundii* antigen. This line was identical with that produced between *A. naeslundii* antigen and antiserum (Fig. 1B). No precipitin reactions occurred between either *A. bovis* antisera and *A. israelii* antigens or *A. bovis* antigens and *A. israelii* antisera. This indicated a serological separation of the two species, in so far as the systems employed in this report could detect (Fig. 1C). There were no precipitin bands formed between the *Actinomyces* antigens and antisera prepared against "anaerobic diphtheroids," or between diphtheroid antigens and *Actinomyces* antisera (Fig. 1B and 1C).

The results shown in Table 2 indicate that all *A. israelii* isolates shared common antigens. The *A. bovis* isolates were serologically distinct from *A. israelii*. *A. naeslundii* shared a minor component with both *A. israelii* and *A. bovis*. The "anaerobic diphtheroids" were antigenically heterologous and showed no cross reactions with any of the *Actinomyces* species.

Discussion

Before proceeding with the gel diffusion studies presented in this paper, we investigated two other methods of preparing antigens: supernatant culture fluid concentrated by evaporation, and acid-soluble extracts. Of these, the concentrated supernatant culture fluid appeared to offer promise of greater specificity, although the antigenic concentration proved to be low. Since the acid-soluble extracts produced no zones of precipitation, or very weak zones, we discarded this method. Finally, acetone concentrates of the supernatant culture fluid provided specific antigens which produced sharp lines. Consequently, we employed this method throughout our work.

It is probable that this acetone-precipitated antigen consists primarily of extracellular polysaccharides from the cell wall. Kwapinski (1960), investigating the antigenic analysis of *A. israelii*, observed that only a polysaccharide fraction from the cell wall displayed serological type specificity. In further studies with *A. israelii* and *Mycobacterium* species, Kwapinski and Snyder (1961) showed that species and type specificity are characterized by polysaccharide components of the cell wall, whereas polysaccharide and protein constituents of cytoplasm bear some common or related antigenic characteristics.

Cummins and Harris (1956*a*, *b*; 1958; 1959) made a comparison between the chemical composition of the cell walls of *A. israelii*, *A. bovis*, and several *Corynebacterium* species. The majority of their *A. israelii* isolates had identical cell-wall composition (i.e., galactose, glucosamine, muramic acid, alanine, glutamic acid, and lysine), and formed a serologically homogenous group. On the other hand, the cell-wall pattern of the *A. bovis* strains differed as to amino acid content, and contained the distinctive sugars rhamnose, fucose, and an unidentified sugar "P."

The antigenic composition of the same cell-wall fractions was analyzed by Cummins (1962) utilizing the agglutination test, the results of which correlated very well with previous cell-wall studies.

Thus, our findings using gel diffusion technique are in agreement with those of Erikson (1940) and Cummins (1962) in establishing species specificity for *A. israelii* and *A. bovis*, although those investigators used different immunological procedures in their work. But we disagree with Slack et al. (1961), who established serological groups unrelated to the habitat and source.

By the use of the gel diffusion technique, it has been shown that *A. israelii* is antigenically distinct from *A. bovis*, whereas *A. naeslundii* cross-reacts with both *A. israelii* and *A. bovis*. This suggests that *A. naeslundii* is a transitional form, exhibiting antigenic relationship to the two other species. Although the groups tested were small, the results warrant investigation to enlarge upon our findings.

Acknowledgments

The authors gratefully acknowledge the interest, advice, and criticism of Arthur V. Kroeger and Abraham S. Markowitz.

Financial support for this investigation was provided by the Graduate College Research Fund, University of Illinois.

Literature Cited

Cummins, C. S. 1962. Chemical composition and antigenic structure of cell walls of *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Actinomyces*, and *Arthrobacter*. J. Gen. Microbiol. **28**:35–50.

Cummins, C. S., and H. Harris. 1956*a*. The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character. J. Gen. Microbiol. **14**:583–600.

Cummins, C. S., and H. Harris. 1956*b*. A comparison of cell wall composition in *Nocardia*, *Actinomyces*, *Mycobacterium*, and *Propionibacterium*. J. Gen. Microbiol. **15**:ix.

Cummins, C. S., and H. Harris. 1958. Studies on the cell-wall composition and taxonomy of *Actinomycetales* and related groups. J. Gen. Microbiol. **18**:173–189.

Cummins, C. S., and H. Harris. 1959. Cell wall composition in strains of *Actinomyces* isolated from human and bovine lesions. J. Gen. Microbiol. **21**:ii.

Erikson, D. 1940. Pathogenic anaerobic organisms

of the *Actinomyces* group. Med. Res. Council, Spec. Rept. Ser. No. 240, p. 1–63.

GOYAL, R. K. 1938. A serological study of the actinomycetes. Indian J. Med. Res. **25**:843–846.

HEINER, D. C. 1958. Diagnosis of histoplasmosis using precipitin reactions in agar gel. Pediatrics **22**:616–627.

HOWELL, A., JR., W. C. MURPHY, F. PAUL, AND R. M. STEPHAN. 1959. Oral strains of *Actinomyces*. J. Bacteriol. **78**:82–95.

KADEN, R. 1956. Precipitation of Sporotrichum antiserum in agar medium. Z. Haut-Geschlechtskrankh. **21**:87–95.

KING, S., AND E. MEYER. 1957. Metabolic and serologic differentiation of *Actinomyces bovis* and "anaerobic diphtheroids." J. Bacteriol. **74**:234–238.

KROEGER, A. V., AND L. R. SIBAL. 1961. Biochemical and serological reactions of an oral filamentous organism. J. Bacteriol. **81**:581–585.

KWAPINSKI, J. B. 1960. Researches on the antigenic structure of *Actinomycetales*. IV. Chemical and antigenic structure of *Actinomyces israelii*. Pathol. Microbiol. **23**:158–172.

KWAPINSKI, J. B., AND M. L. SNYDER. 1961. Antigenic structure and serological relationships of *Mycobacterium*, *Actinomyces*, *Streptococcus*, and *Diplococcus*. J. Bacteriol. **82**:632–639.

LENTZE, F. A. 1938. Zur Bakteriologie und Vakzinetherapie der Aktinomykose. Zentr. Bakteriol. Parisitenk., Abt I, Orig. **141**:21–36.

NAESLUND, C. 1925. Studies on *Actinomyces* from the oral cavity. Acta Pathol. Microbiol. Scand. **2**:110–140.

OUCHTERLONY, O. 1949. Antigen-antibody reaction in gels. Acta Pathol. Microbiol. Scand. **26**:507.

SCHUBERT, J. H., H. J. LYNCH, JR., AND L. AJELLO. 1961. Evaluation of the agar-plate precipitin test for histoplasmosis. Am. Rev. Respirat. Diseases **84**:845–849.

SEELIGER, H. 1956. A serological study of *Hyphomycetes* causing mycetoma in man. J. Invest. Dermatol. **26**:81–93.

SLACK, J. M., R. G. SPEARS, W. G. SNODGRASS, AND R. J. KUCHLER. 1955. Studies with microaerophilic actinomycetes. II. Serological groups as determined by the reciprocal agglutinin adsorption technique. J. Bacteriol. **70**:400–404.

SLACK, J. M., A. WINGER, AND D. W. MOORE, JR. 1961. Serological grouping of *Actinomyces* by means of fluorescent antibodies. J. Bacteriol. **81**:54–65.

SUTER, L. S. 1956. Evaluation of criteria used in the identification of *Actinomyces bovis* with particular reference to the catalase reaction. Mycopathol. Mycol. Appl. **7**:220.

THOMPSON, L., AND B. A. LOVESTEDT. 1951. An *Actinomyces*-like organism obtained from the human mouth. Proc. Staff Meetings Mayo Clinic **26**:169–175.