Identification of *Propionibacterium acnes* and Related Organisms by Precipitin Tests with Trichloroacetic Acid Extracts

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The serological identification of *Propionibacterium acnes*, *P. granulosum*, and *P. avidum*, using trichloroacetic acid extracts, is described. With antisera prepared against reference strains, the method has been tested on 142 strains recently isolated from human skin. All except two of the strains could be identified serologically, and there was excellent agreement between the serological results and the fermentation pattern of the strains. Two serological types of *P. acnes* and two of *P. avidum* were identified, but only one of *P. granulosum*.

Anaerobic or microaerophilic coryneform organisms comprise a large part of the skin flora in man, the most common member of the group probably being the organism traditionally known as *Corynebacterium acnes*. However, these anaerobic coryneforms differ in a number of major characters from the classical corynebacteria as exemplified by *C. diphtheriae* (7), and in the latest edition of *Bergey's Manual* (10) they have been transferred to the genus *Propionibacterium*.

A number of different antigenic types have been recognized among these organisms. For example, Linzenmaier (8, 9) recognized four groups by agglutination, using antisera prepared against strains identified as *Corynebacterium diphtheroides*, *C. granulosum*, *C. parvum*, and *C. acnes*. Pulverer and Ko (11), also using agglutination tests, could distinguish 11 different serotypes among 72 strains labeled *Propionibacterium acnes*. Other techniques, including passive hemagglutination (1), latex agglutination (5), and immunofluorescence (6), have also indicated the presence of different serological types among these organisms. By precipitin tests, using whole broth cultures as antigens, Voss (15) was able to distinguish two groups among 143 strains of anaerobic coryneforms from skin and acne lesions. The two groups also differed in physiological properties.

Slide agglutination tests have been used by a number of investigators (e.g., 12) for the identification of *P. acnes* (*C. acnes*), especially since antisera are commercially available. However, a number of strains may be autoagglutinable, which makes the method unreliable.

Previous studies in this laboratory (4, 7) have shown that a number of different serological

types, based on cell wall (CW) polysaccharide antigens, can be recognized among the anaerobic coryneforms. These types were originally distinguished by CW agglutination tests. However, the need to prepare CW suspensions makes CW agglutination too slow for routine purposes. Various acid extraction techniques therefore were tried, to find a faster and more economical method of obtaining the CW polysaccharide antigens in a useful form.

This paper describes a trichloroacetic acid extraction procedure which has been found to be satisfactory.

MATERIALS AND METHODS

Strains. (i) Origins of the strains used to produce antisera. *P. acnes*, type I, strain VPI 3706, was from human skin. This strain was previously described (7) as having been isolated from feces, but this was an error; the strain was originally isolated in a NASA investigation and came from the skin of an astronaut. *P. acnes*, type II, strain VPI 0162 is Prévot strain *C. anaerobium* 578; the original source is not known. *P. granulosum*, strain VPI 0507 was isolated as a contaminant from a culture labeled *Staphylococcus aerogenes*. *P. avidum*, type I, strain VPI 0575 is CDC strain 71, isolated from a hip sinus. *P. avidum*, type II, strain VPI 0589 is CDC strain A518, isolated from a wound swab.

(ii) Other strains. A number of other strains from skin and other sources have been tested to check the correlation between physiological characters and serological grouping as determined by trichloroacetic acid extracts. The strains selected were recent isolates from skin. Their origins are described below.

Preparation of antisera. Rabbits were injected intravenously with purified CW preparations in saline (5 mg of CW/ml). Two injections (1 ml each) were given each week for 3 weeks, and the sera were tested 1 week after the last injection. If satisfactory

(titer of 1/320 or above in CW agglutination tests), the animals were bled out from the heart. If the titers were unsatisfactory, the animals were rested for 1 to 2 months and given a second course of injections.

Concentration of antisera. Antisera were concentrated approximately threefold using Lyphogel (Gelman Instrument Co.): 0.2 g of Lyphogel was added to serum for each 1 ml of water to be removed, the mixture was held overnight in the refrigerator, and the serum was recovered by centrifugation. It is convenient to centrifuge the swollen Lyphogel granules in a small plastic tube (18 by 100 mm) with holes bored at one end with a hot wire. If this is held inside a larger glass tube and centrifuged at low speed (ca. $500 \times g$), the yield of concentrated serum is considerably increased.

Preparation of antigen extracts. Polysaccharide antigens were extracted from approximately 10 mg (dry weight) of washed cells by resuspending the cells in 1 ml of 10% trichloroacetic acid and heating the mixture in a water bath at 56 C for 30 min with occasional shaking. After cooling, the mixture was centrifuged and 5 volumes of acetone was added to the supernatant, together with 2 drops of a 5% potassium acetate solution. After several hours (or overnight) at 4 C, the precipitates were recovered by centrifugation, redissolved in 1 ml of distilled water, and neutralized to phenol red with N/20 KOH. Insoluble material was finally removed by centrifugation. In a number of cases, CWs were extracted, in which case approximately 5-mg amounts of CWs were used in the extraction procedures described above.

Immunodiffusion tests. These were done in 1% Ionagar, buffered to pH 7.5 with 0.05 M phosphate in 0.9% NaCl. The reagent wells were 5 mm in diameter, with centers 10 mm apart. Tests were incubated at room temperature (22 to 24 C) in a water-saturated atmosphere.

Absorption of antisera. Antisera were absorbed with CW suspensions or in some cases with polysaccharides which had been extracted with trichloroacetic acid as described above. In the latter case, the antigen solutions were lyophilized so that absorption could be done with a known weight of material.

(i) **Absorption with CW suspensions.** This was done as previously described (7), except that sera were usually absorbed at a dilution of 1 in 5 and treated with Lyphogel after absorption to give a final concentration about three times that of the original serum.

(ii) **Absorption with polysaccharide.** This was done by adding the appropriate amount (see below) of polysaccharide in solution in saline to an equal volume of undiluted serum. The mixture was incubated at 37 C for 1 to 2 h and then held at 4 C for 2 days, after which the precipitate was centrifuged out. The serum was then usually concentrated $\times 3$ (based on the original volume of serum) with Lyphogel. Preliminary tests were done by adding varying amounts of polysaccharide to 1 ml of serum to determine the level (milligrams of polysaccharide/milliliter of serum) which would just fail to leave

detectable excess in the absorbed serum. As an example of the amount required, it was found that if more than 0.3 mg of 0162 polysaccharide per ml was added to a particular 3706 antiserum, excess could be detected when the absorbed serum was tested against 0162 antiserum by immunodiffusion.

Preparation of CW fractions. This was done as previously described (2).

Paper chromatography. Descending chromatography on Whatman no. 1 paper was used, and two solvent systems were employed: (i) ethyl acetate, pyridine, water (80:20:10); (ii) butanol, pyridine, 0.4% acetic acid in water (60:35:25). Chromatograms were developed in solvent (i) for 18 h for hexoses, and in solvent (ii) for 36 to 48 h for separation of hexosamines. Spots were visualized with aniline phthalate (hexoses) or alkaline silver (hexosamines) (13, 14).

RESULTS AND DISCUSSION

Methods of extraction and properties of the extracts. The method of extraction eventually chosen, 10% trichloroacetic acid at 56 C for 30 min seemed simple, convenient, and reasonably quick and produced satisfactory antigenic preparations from all strains tested. In general trichloroacetic acid at 80 C (15 min) appeared to produce very similar results, but in some cases the precipitin lines were weaker, as though some destruction of antigen had occurred. Equally good preparations could be obtained with 10% trichloroacetic acid at 37 C, but the extraction period had to be more prolonged (several hours or overnight). At 4C, very little material was extracted by trichloroacetic acid in 24 h.

Immunologically similar material could be extracted by formamide at 165 C (15 min) and by 1% acetic acid at 100 C (15 min). Antigenic material could also be obtained by using N/20 HCl at 100 C for 15 min, but the HCl extracts did not always give as many lines, and these were often more diffuse, suggesting that there had been excessive breakdown of the material.

The material extracted by trichloroacetic acid at 56 C from the "standard" strains (those used to prepare antisera) was hydrolyzed and examined by paper chromatography and the results are shown in Table 1. The trichloroacetic acid-extracted polysaccharides contained the same sugars as had been found in CW hydrolysates of the extracted strains (7). In addition to examination for hexoses, the extracted polysaccharides were also examined for hexosamines, which had not been done previously on the CW hydrolysates.

Characteristics of reactions in different groups. (i) *P. acnes*. It has been shown (7) that there are two serological types of *P. acnes*,

TABLE 1. Sugar components in CW polysaccharides of anaerobic coryneforms

Strain	Designation	Hexoses	Hexosamines ^a	
			Hydrolysis (2 h)	Hydrolysis (18 h)
3706	<i>Propionibacterium acnes</i> type I	Galactose, glucose, mannose	ND	Galactosamine
0162	<i>P. acnes</i> type II	Glucose, mannose	Galactosamine	Galactosamine (glucosamine, trace)
0575	<i>P. avidum</i> type I	Galactose, glucose, mannose	Galactosamine	Galactosamine, glucosamine
0589	<i>P. avidum</i> type II	Glucose, mannose	Galactosamine	Galactosamine
0507	<i>P. granulosum</i>	Galactose, mannose (plus trace glucose)	ND	nd

^a ND, None detected; nd, not done.

which are referred to as *P. acnes* type I and *P. acnes* type II. These types were originally identified by differences in CW sugar patterns and by serological reactions using CW agglutination tests. The two types can also be recognized using trichloroacetic acid extracts, although there is considerable cross-reaction between them with unabsorbed sera, as illustrated in Fig. 1. Extracts of all six strains (three type I and three type II) gave what appeared to be a single line with the type I antiserum (Fig. 1a). With the type II serum (Fig. 1b), the type II strains (wells D, E, and F) gave two or three lines whereas the type I strains gave only a single rather weak line. With type I serum absorbed with type II polysaccharide, however, only the type I strains reacted (Fig. 1c). These reactions are well-developed in 18 to 24 h at room temperature.

A comparison of the results with absorbed and unabsorbed type I serum suggests that the apparent single line given by all six strains with unabsorbed serum is in reality a composite reaction involving two or more antigen-antibody systems, only one of which is specific for type I strains.

(ii) *P. granulosum*. The reactions of extracts of *P. granulosum* strains with the standard *granulosum* serum (against strain 0507) regularly showed two distinct lines with possibly a third indistinct line close to the serum well (Fig. 2a and b). It is also a characteristic feature

FIG. 1. Reactions of *P. acnes* type I and II strains. Results at 24 h (a) against 3706 serum (type I); (b) against 0162 serum (type II); and (c) against 3706 serum, absorbed with 0162 polysaccharide. S, Serum. (A, B, C) Type I extracts (three different strains); (D, E, F) Type II extracts (three different strains).

FIG. 2. Reactions of *P. granulosum* strains (a) at 24 h and (b) at 40 h [same plate as (a)]. S, 0507 serum (*P. granulosum*). (A, C, E) 0507 CW extract; (B, D, F) *P. granulosum* whole cell extracts (three different strains).

of the reactions of this group that they are slower to develop under standard conditions than those of *P. acnes* or *P. avidum* and lines may not develop fully for 48 to 72 h, or even longer. This relatively slow development may be seen by comparing the results at 24 h (Fig. 2a) and 40 h (Fig. 2b).

The number of strains (26) of *P. granulosum* so far examined is considerably smaller than that of *acnes*, but there is no clear evidence as yet of more than one serological type in *P. granulosum*.

(iii) *P. avidum*. By comparison with *P. acnes*, the two serological types of *P. avidum* did not show any marked degree of cross-reaction, and each reacted with its serum in a rather distinctive way. The strains which are here referred to as *P. avidum* type I showed a single line with 0575 serum, which was used as the reference serum for this type (Fig. 3). These reactions were generally well-developed at about 24 h under the conditions described.

The type II strains of *P. avidum* on the other

hand usually showed a characteristic pattern of two lines with the type serum (to strain 0589) as shown in Fig. 4. However, two strains which otherwise fall into this group showed only one of these lines, that nearer the serum well, as illustrated by the reaction of strain 0670 (Well D, Fig. 4).

It appears that loss of this antigen may occur rather readily in these strains. For example, cultures of strain 4982 were sent to another laboratory several years ago, and subcultures were recently received from there for comparison with the original. When extracts of this subculture [4982 (ii): wells A and B in Fig. 5b] were compared with extracts of the original culture maintained at Virginia Polytechnic Institute [4982 (i): wells C and D in Fig 5b], it was found that they differed with respect to this

FIG. 3. Reactions of *P. avidum* type I strains. Results at 24 h. S, 0575 serum (*P. avidum* I). (A, C, E) 0575 CW extracts; (B, D, F) *P. avidum* whole cell extracts (three different strains).

FIG. 4. Reactions of *P. avidum* type II strains. Results at 24 h. S, 0589 serum (*P. avidum* II). (A, C, E) 0589 CW extracts; (B, D, F) *P. avidum* whole cell extracts (three different strains).

FIG. 5. Cross reaction between *P. acnes* type II and *P. avidum* type II. Results at 24 h. (a) S, 0162 serum. (A, B) Extracts of 4982 (i) (*avidum* II); (C, D) Extracts of 4982 (ii) (*avidum* II); (E) 0162 polysaccharide (*acnes* II); (F) 0589 polysaccharide (*avidum* II). (b) S, 0589 serum. Extracts as in (a). For derivation of strains 4982 (i) and 4982 (ii) see text.

outer line which is now absent from extracts of the VPI cultures. The reasons for their apparent loss of antigen are not yet known. The reactions of *avidum* type II strains usually develop more rapidly than those of *avidum* type I and are often well-marked at 18 to 20 h.

Cross-reactions between *P. acnes* type II and *P. avidum* type II. There is often some degree of cross-reaction between extracts of *P. acnes* type II and *P. avidum* type II serum, or vice versa. This is especially the case with concentrated sera, and is illustrated by the reactions shown in Fig. 5a and b.

There are a number of similarities between strains of *P. acnes* type II and *P. avidum* type II. For example, occasional strains in both these groups have *meso*-DAP instead of the *L*-isomer usual in the propionibacteria, and in both galactose is absent from the CW polysaccharide. However, taking into account the reactions of each kind of strain with its homologous serum, and their different fermentation patterns, these serological cross-reactions do not cause difficulties in identifying strains.

Correlation between antigenic composition and other characteristics of strains. The strains originally used to make antisera were

chosen as representative of different deoxyribonucleic acid homology groups, or of serological types within these groups. However, their origins were somewhat atypical since only one came from skin. Subsequent investigations (4, 7), using CW agglutination tests, also dealt with strains which might be regarded as rather atypical, since many came from patients suffering from leukemia or other diseases of the reticulo-endothelial system. It was decided therefore to investigate a series of strains of anaerobic diphtheroids known to have been isolated from skin, since this represents one of the major normal habitats for these organisms in man. We wished to determine the degree of correlation between serological grouping and fermentation pattern, and also to see whether there was any large group of strains which failed to react with any of the available sera, thus indicating the possible presence of other serological types.

In all, 142 skin strains have been examined serologically (trichloroacetic acid extracts) and by fermentation tests. The strains came from 10 individuals in Blacksburg, Va., and 15 in Seattle, Wash., and the great majority (133 strains) were from the skin of the forehead. Of the others, two were from the deltoid region, two from the back, two from the axilla, and three from acne comedones. The fermentation tests used and the reaction patterns expected with *P. acnes*, *P. granulosum*, and *P. avidum* have been detailed before (4); they are summarized here in Table 2.

Approximately 80% of the strains examined (116 strains) were identified by overall characters as *P. acnes* and 20% (26 strains) as *P. granulosum*. However, this should not be taken as representing the relative distribution of these organisms on the skin, since the strains from Seattle had been specially selected on the basis of colonial morphology before being sent to Blacksburg for further tests.

The correlation between serological and physiological tests is summarized in Table 3. In the case of the *acnes* strains the correlation was extremely good and only two strains regarded as *P. acnes* serologically differed from the normal fermentation pattern: these two strains did not produce indole. Conversely, only one strain which had a typical *acnes* fermentation pattern failed to react with either of the *acnes* type antisera. Approximately 70% of the *P. acnes* strains were serologically type I and 30% type II, and of the type I strains 80% fermented sorbitol. This carbohydrate is apparently not attacked by type II strains, so that it can be assumed that sorbitol-positive strains belong to type I.

Of the presumptive *P. granulosum* strains

TABLE 2. Typical reaction patterns of anaerobic coryneforms^a

Identification	Glucose	Sorbitol	Maltose	Sucrose	Esculin ^b	Indole	NO ₃	Gelatine
<i>Propionibacterium acnes</i> I	+ ^c	+ or -	-	-	-	+	+	+
<i>P. acnes</i> II	+	-	-	-	-	+	+	+
<i>P. granulosum</i>	+	-	+	+	-	-	-	-
<i>P. avidum</i>	+	-	+	+	+	-	-	+

^a For details of interpretation of tests, see (4).

^b In the case of esculin, + indicates a positive ferric citrate test. The pH in these tubes did not change significantly.

^c +, Fermentation of the carbohydrate in question or (in the last three columns) the production of indole, reduction of nitrate to nitrite, or the liquefaction of gelatine, respectively.

TABLE 3. Summary of results in 142 strains of anaerobic diphtheroids isolated from skin

Identification	No. of strains	Sorbitol hydrolysis		No. of atypical strains	Characters of atypical strains
		+	-		
<i>Propionibacterium acnes</i> type I	80 (69%)	64 (80%)	16 (20%)	3	Two strains were indole negative, but otherwise typical
<i>P. acnes</i> type II	36 (31%)		36		One strain did not react with either type I or type II serum, but was otherwise typical
<i>P. granulosum</i>	116 (100%) 26		26	12	Four strains did not ferment sucrose and/or maltose; five strains reduced NO ₃ ; three strains produced indole and/or liquefied gelatine (one of these three also reduced NO ₃); one strain did not react with 0507 serum

almost half (12 out of 26 strains) differed in some way from the normal fermentation pattern as given in Table 2, mostly by failing to ferment sucrose and/or maltose, or by producing indole and/or reducing nitrate. However, only one of the 26 strains failed to react with 0507 serum. The original differentiation of *P. granulosum* from *P. acnes* on grounds of deoxyribonucleic acid homology (7) was based on a rather small number of strains, but there is no doubt that *granulosum* strains can be distinguished from *acnes* strains serologically and by fermentation tests. This was also the conclusion of Voss (15) who used similar tests, although he referred to these strains (i.e., *P. granulosum*) as *C. acnes* group II.

It is of interest that none of these 142 skin strains fell into the *avidum* group either serologically or by fermentation tests. We have not so far been able to examine any large collection of strains of *P. avidum* and most of those available to us have come from suppurative lesions. It would appear that these organisms are not common among the skin flora, although they cer-

tainly do occur there (e.g., strain 4982, see reference 7). It may be noted also that all but two of the 142 strains could be identified serologically as belonging either to *P. granulosum* or to one of the two types of *P. acnes*. This suggests that other serological varieties, if they exist, must be infrequent, at least on the skin. However, recent work by Marples and McGinley (9a) suggests that *P. avidum* may be relatively common in the axilla.

We have not made any systematic comparison between the results with trichloroacetic acid extracts and those obtained by other serological techniques. In a limited series of tests using slide agglutination and the same antisera as were used in the trichloroacetic acid extract precipitin tests, it was generally possible to recognize *acnes*, *granulosum*, or *avidum* strains, and to distinguish between *P. avidum* type I and *P. avidum* type II. There was a great deal of cross-reaction between type I and type II *acnes* strains, and also cross-reaction between strains of *acnes* II and *avidum* II. In these tests the commercially available antisera for *P.*

acnes (Difco 554 and 605) reacted in the same way as our own sera, that is, all the strains of *P. acnes* tested reacted with one serum or the other, and usually with both. This is to be expected, since strain 554 is a *P. acnes* type I and strain 605 is *P. acnes* type II (7).

We did not find any evidence of more than two types among *P. acnes* strains. This is in agreement with our previous results (7) and is also in accord with the results of Holmberg and Forsum (6) using immunofluorescence. The fact that fluorescent antibody techniques will pick up the same antigenic determinants as are found in trichloroacetic acid extracts indicates that some at least of the active material is present at the surface of the cell. However, it appears that other antigenic material must also be present at the cell surface since the results of Pulverer and Ko (11) show that there may be up to 11 different agglutinating types among *P. acnes* strains.

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