

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) SERUM ANTIBODY TO PSEUDOMONAS AERUGINOSA EXOTOXIN MEASURED BY A PASSIVE HEMAGGLUTINATION ASSAY.		5. TYPE OF REPORT & PERIOD COVERED MEDICAL RESEARCH PROGRESS REPORT
6. AUTHOR(s) MATTHEW POLLACK NANCY S. TAYLOR		7. PERFORMING ORG. REPORT NUMBER
9. PERFORMING ORGANIZATION NAME AND ADDRESS NAVAL MEDICAL RESEARCH INSTITUTE BETHESDA, MARYLAND 20014		8. CONTRACT OR GRANT NUMBER(s) MED-111
11. CONTROLLING OFFICE NAME AND ADDRESS NAVAL MEDICAL RESEARCH & DEVELOPMENT COMMAND BETHESDA, MARYLAND 20014		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS MR041 20 01, 0423 Report No. 3
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) BUREAU OF MEDICINE & SURGERY DEPARTMENT OF THE NAVY WASHINGTON, D. C. 20372		12. REPORT DATE JULY, 1977
		13. NUMBER OF PAGES 4
		15. SECURITY CLASS. (of this report) UNCLASSIFIED
16. DISTRIBUTION STATEMENT (of this Report) APPROVED FOR PUBLIC RELEASE AND SALE; DISTRIBUTION UNLIMITED. -16p-		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) DDC RECEIVED FEB 13 1979 RECEIVED		
18. SUPPLEMENTARY NOTES Journal of Clinical Microbiology, Vol. 6, p. 58-61, 1977		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) PSEUDOMONAS aeruginosa, Passive Hemagglutination Assay (PHA), Toxin, Cyto- toxicity neutralization (CTN) and 2-mercaptoethanol (2-ME).		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A passive hemagglutination (PHA) assay for antibody to Pseudomonas aeruginosa exotoxin is described which utilizes chromic chloride-treated ovine erythrocytes coated with purified toxin. PHA antitoxin titers correlated well with those obtained by a cytotoxicity neutralization assay ($r = 0.91$, $P < 0.001$), whereas the PHA assay was four to eight times as sensitive. This mean serum PHA titer of 16 patients convalescing from recent pseudomonas infections ($\log_2 = 9.4 \pm 3.9$) was significantly higher ($P < 0.001$) than that of 17 healthy		

DD FORM 1473

1 JAN 73

EDITION OF 1 NOV 65 IS OBSOLETE
S/N 0102-014-6601

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

DDC FILE COPY, A064626

**Best
Available
Copy**

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

ACROSS: 1. for
2. 18
3. 18
4. 18
5. 18
6. 18
7. 18
8. 18
9. 18
10. 18
11. 18
12. 18
13. 18
14. 18
15. 18
16. 18
17. 18
18. 18
19. 18
20. 18
21. 18
22. 18
23. 18
24. 18
25. 18
26. 18
27. 18
28. 18
29. 18
30. 18
31. 18
32. 18
33. 18
34. 18
35. 18
36. 18
37. 18
38. 18
39. 18
40. 18
41. 18
42. 18
43. 18
44. 18
45. 18
46. 18
47. 18
48. 18
49. 18
50. 18
51. 18
52. 18
53. 18
54. 18
55. 18
56. 18
57. 18
58. 18
59. 18
60. 18
61. 18
62. 18
63. 18
64. 18
65. 18
66. 18
67. 18
68. 18
69. 18
70. 18
71. 18
72. 18
73. 18
74. 18
75. 18
76. 18
77. 18
78. 18
79. 18
80. 18
81. 18
82. 18
83. 18
84. 18
85. 18
86. 18
87. 18
88. 18
89. 18
90. 18
91. 18
92. 18
93. 18
94. 18
95. 18
96. 18
97. 18
98. 18
99. 18
100. 18
101. 18
102. 18
103. 18
104. 18
105. 18
106. 18
107. 18
108. 18
109. 18
110. 18
111. 18
112. 18
113. 18
114. 18
115. 18
116. 18
117. 18
118. 18
119. 18
120. 18
121. 18
122. 18
123. 18
124. 18
125. 18
126. 18
127. 18
128. 18
129. 18
130. 18
131. 18
132. 18
133. 18
134. 18
135. 18
136. 18
137. 18
138. 18
139. 18
140. 18
141. 18
142. 18
143. 18
144. 18
145. 18
146. 18
147. 18
148. 18
149. 18
150. 18
151. 18
152. 18
153. 18
154. 18
155. 18
156. 18
157. 18
158. 18
159. 18
160. 18
161. 18
162. 18
163. 18
164. 18
165. 18
166. 18
167. 18
168. 18
169. 18
170. 18
171. 18
172. 18
173. 18
174. 18
175. 18
176. 18
177. 18
178. 18
179. 18
180. 18
181. 18
182. 18
183. 18
184. 18
185. 18
186. 18
187. 18
188. 18
189. 18
190. 18
191. 18
192. 18
193. 18
194. 18
195. 18
196. 18
197. 18
198. 18
199. 18
200. 18
201. 18
202. 18
203. 18
204. 18
205. 18
206. 18
207. 18
208. 18
209. 18
210. 18
211. 18
212. 18
213. 18
214. 18
215. 18
216. 18
217. 18
218. 18
219. 18
220. 18
221. 18
222. 18
223. 18
224. 18
225. 18
226. 18
227. 18
228. 18
229. 18
230. 18
231. 18
232. 18
233. 18
234. 18
235. 18
236. 18
237. 18
238. 18
239. 18
240. 18
241. 18
242. 18
243. 18
244. 18
245. 18
246. 18
247. 18
248. 18
249. 18
250. 18
251. 18
252. 18
253. 18
254. 18
255. 18
256. 18
257. 18
258. 18
259. 18
260. 18
261. 18
262. 18
263. 18
264. 18
265. 18
266. 18
267. 18
268. 18
269. 18
270. 18
271. 18
272. 18
273. 18
274. 18
275. 18
276. 18
277. 18
278. 18
279. 18
280. 18
281. 18
282. 18
283. 18
284. 18
285. 18
286. 18
287. 18
288. 18
289. 18
290. 18
291. 18
292. 18
293. 18
294. 18
295. 18
296. 18
297. 18
298. 18
299. 18
300. 18
301. 18
302. 18
303. 18
304. 18
305. 18
306. 18
307. 18
308. 18
309. 18
310. 18
311. 18
312. 18
313. 18
314. 18
315. 18
316. 18
317. 18
318. 18
319. 18
320. 18
321. 18
322. 18
323. 18
324. 18
325. 18
326. 18
327. 18
328. 18
329. 18
330. 18
331. 18
332. 18
333. 18
334. 18
335. 18
336. 18
337. 18
338. 18
339. 18
340. 18
341. 18
342. 18
343. 18
344. 18
345. 18
346. 18
347. 18
348. 18
349. 18
350. 18
351. 18
352. 18
353. 18
354. 18
355. 18
356. 18
357. 18
358. 18
359. 18
360. 18
361. 18
362. 18
363. 18
364. 18
365. 18
366. 18
367. 18
368. 18
369. 18
370. 18
371. 18
372. 18
373. 18
374. 18
375. 18
376. 18
377. 18
378. 18
379. 18
380. 18
381. 18
382. 18
383. 18
384. 18
385. 18
386. 18
387. 18
388. 18
389. 18
390. 18
391. 18
392. 18
393. 18
394. 18
395. 18
396. 18
397. 18
398. 18
399. 18
400. 18
401. 18
402. 18
403. 18
404. 18
405. 18
406. 18
407. 18
408. 18
409. 18
410. 18
411. 18
412. 18
413. 18
414. 18
415. 18
416. 18
417. 18
418. 18
419. 18
420. 18
421. 18
422. 18
423. 18
424. 18
425. 18
426. 18
427. 18
428. 18
429. 18
430. 18
431. 18
432. 18
433. 18
434. 18
435. 18
436. 18
437. 18
438. 18
439. 18
440. 18
441. 18
442. 18
443. 18
444. 18
445. 18
446. 18
447. 18
448. 18
449. 18
450. 18
451. 18
452. 18
453. 18
454. 18
455. 18
456. 18
457. 18
458. 18
459. 18
460. 18
461. 18
462. 18
463. 18
464. 18
465. 18
466. 18
467. 1

~~CONFIDENTIAL~~ CLASSIFICATION OF THIS PAGE: ~~CONFIDENTIAL~~ - DNA EXAMINED

Serum Antibody to *Pseudomonas aeruginosa* Exotoxin Measured by a Passive Hemagglutination Assay¹

MATTHEW POLLACK* AND NANCY S. TAYLOR

Department of Microbiology, Naval Medical Research Institute, Bethesda, Maryland 20014

Received for publication 25 February 1977

A passive hemagglutination (PHA) assay for antibody to *Pseudomonas aeruginosa* exotoxin is described which utilizes chromic chloride-treated ovine erythrocytes coated with purified toxin. PHA antitoxin titers correlated well with those obtained by a cytotoxicity neutralization assay ($r = 0.91$, $P < 0.001$), whereas the PHA assay was four to eight times as sensitive. The mean serum PHA titer of 16 patients convalescing from recent pseudomonas infections ($\log_2 = 9.4 \pm 3.9$) was significantly higher ($P < 0.001$) than that of 17 healthy controls (2.8 ± 2.6), and fourfold or greater rises were demonstrated in 5 of 7 patients examined sequentially. The lower levels of PHA antibody in sera from 11 of 17 controls suggested the acquisition of antitoxin secondary to asymptomatic infection.

A pathogenic role is suspected for the exotoxin produced in vitro by the majority of clinical *Pseudomonas aeruginosa* strains representing all immunotypes (5). The demonstration of serum antitoxin in high titer in convalescent patients by a previously described cytotoxicity neutralization (CTN) assay indicates the in vivo release of exotoxin during pseudomonas infections (4). The CTN assay requires tissue culture facilities, the use of a radioactive label, and several days to perform.

We describe here a new passive hemagglutination (PHA) assay for antibody to pseudomonas exotoxin that utilizes chromic chloride-treated ovine erythrocytes (SRBC) sensitized with purified toxin. This assay is simpler, more rapid, and more sensitive than the CTN assay and yields parallel results. Its applicability in the study of serum antitoxin in patients is demonstrated, and its possible diagnostic usefulness as a substitute for the CTN assay is suggested.

MATERIALS AND METHODS

Toxin. Exotoxin produced by *P. aeruginosa* PA 103 was purified as previously described (1, 2). The material used to coat SRBC migrated as a single band on analytical polyacrylamide gel electrophoresis, yielded a single precipitin line when reacted with antiserum to crude toxin by immunodiffusion, and had a 60-ng mean lethal dose for 20-g mice.

Antitoxin sera. Rabbit hyperimmune antitoxin sera were produced as described (4). An adult male goat and sheep each received six weekly intravenous

doses of 25 μ g of purified exotoxin and a single subcutaneous booster dose consisting of 50 μ g of toxin in Freund incomplete adjuvant. Exotoxin-neutralizing antibody was measured by a CTN assay (4). The hyperimmune sheep serum was employed as a reference antitoxin-containing serum throughout.

Sensitization and coating of SRBC. Fresh SRBC, 60% (vol/vol) in Alsever solution (Microbiological Associates, Bethesda, Md.), were sensitized using chromic chloride according to the method of Gold and Fudenberg (3), with the following modifications. The ratio of SRBC to 0.05% chromic chloride to antigen in the sensitizing-coating mixture was 1:2:2 (by volume). The sensitized SRBC were suspended in Veronal-buffered saline (1% vol/vol) with 1% bovine serum albumin, pH 7.3 to 7.4 (VBS/BSA) for use in the PHA assay. Unsensitized control cells were prepared in the same manner except that no toxin was present in the sensitizing mixture.

PHA assay. Twofold dilutions of test sera were carried out manually in test tubes to an initial dilution of 1:16, and subsequent dilutions were done in microtiter plates with V-shaped wells using 50- μ l microdiluters (Microbiological Associates), with VBS/BSA as the diluent. An equal volume (50 μ l) of a 1% suspension of sensitized or unsensitized SRBC was then added to each well, the plates were shaken on a Vortex mixer, and the cells were allowed to settle overnight. Hemagglutination end points were read before and after tilting the microtiter plates at an 80° angle for 4 to 5 min. Although there was close correlation between the two plate-reading methods, tilting produced rapid "runoff" of unagglutinated SRBC from the bottoms of wells, in contrast to the compact, stationary buttons produced by agglutinated cells; this procedure allowed clearer end points and slightly greater sensitivity (6). Control wells contained serial dilutions of patients' sera plus unsensitized SRBC, unsensitized or sensitized cells

¹ Naval Medical Research and Development Command, research task no. MR041.20.01.0423.

plus buffer, and serial twofold dilutions of a standard sheep antitoxin-containing reference serum plus sensitized cells.

Patients' sera. Sera were obtained from patients convalescing from pseudomonas infections at the National Naval Medical Center and from the National Institutes of Health, Bethesda, Md. (the latter courtesy of Herbert Y. Reynolds). In all cases, infections were documented by positive blood or other appropriate cultures and clinical data. Serum was inactivated at 56°C for 30 min and then absorbed with unsensitized SRBC to eliminate nonspecific (heterophile) antibodies that were present in low titer in many patients' sera. The latter procedure was performed in the following manner. Fresh SRBC were washed three times in physiological saline, 0.1 ml of packed cells was added to 1.0 ml of serum, and the suspension was incubated at 37°C for 1 h with frequent mixing and then chilled for 1 additional h at 4°C.

Fractionation and 2-mercaptoethanol treatment of sera. Acute and convalescent sera from several patients were fractionated and treated with 2-mercaptoethanol (2-ME) to identify which major immunoglobulin classes PHA antibodies were associated with. Three milliliters of whole serum was dialyzed against 0.14 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, applied to a Sephadex G-200 column (2.5 by 85 cm; Pharmacia, Piscataway, N.J.) and eluted with tris(hydroxymethyl)aminomethane buffer, and the protein content of each 3-ml fraction was estimated by measuring optical density at a wavelength of 280 nm. To 0.5-ml portions from each fraction, representing three major protein peaks, was added 0.5 ml of 0.2 M 2-ME in phosphate-buffered saline, pH 7.3, and the mixture was allowed to incubate at room temperature overnight. 2-ME-treated and untreated fractions were then assayed by PHA for antitoxin.

Statistics. *P* values were determined by a two-tailed Student's *t* test. The linear regression analysis was performed by the method of least squares, and the validity of fit was determined by the linear correlation coefficient *r*. The significance of *r* was computed by a two-tailed Student's *t* test using the statistic $t = (r \sqrt{n-2}) / \sqrt{1-r^2}$.

RESULTS

Sensitization of SRBC was successfully carried out using the chromium chloride method described and small concentrations of purified exotoxin. As indicated in Table 1, the concentration of exotoxin used to sensitize the SRBC was critical; a dilute solution containing 70 µg/ml appeared optimal.

There was considerable variation in the sensitivity of antibody detection by sensitized cells from different sheep over a 32-fold dilution range (Table 2). In contrast, there was rarely more than a twofold change in antibody titer when cells from the same sheep, sensitized at the same time but used on different days or sensitized at different times, were used to ti-

TABLE 1. Effect of concentration of *Pseudomonas aeruginosa* exotoxin used to sensitize SRBC on titer of PHA antibody of sheep antiserum to exotoxin

Toxin concn (µg/ml)	Antibody titer (log ₂)
1,100	2
550	3
275	13
140	15
70	18
35	17
17	15
8	14
4	14
2	2
1	2

TABLE 2. PHA titers of sheep antiserum to exotoxin obtained by using exotoxin-sensitized SRBC from different sheep

Sheep no.	Antibody titer (log ₂)
1	13
2	15
5	16
8	16
17	18

trate a "standard" reference serum. Although all assay results reported here were obtained with cells coated with exotoxin from a single preparation, we have also successfully coated cells that yielded similar titers with exotoxin from two other batches prepared by different purification procedures. Once sensitized, SRBC stored at 4°C would maintain their PHA reactivity for as long as 3 to 4 weeks; after this, hemolysis became noticeable, and titer changes occurred.

After initial screening of erythrocytes from five different sheep revealed that the highest PHA titers were obtained with SRBC from animal no. 17, cells from this sheep were used in all subsequent studies. A "standard" sheep hyperimmune antitoxin was used for all of the above-described tests as well as for a quality control in all tests with human sera.

As shown in Table 3, PHA antibody to exotoxin was present in high titer in sera from hyperimmunized rabbits, sheep, and goat. Titers roughly paralleled those obtained by the CTA assay but were two to eight times higher in various animals (Table 3), indicating the greater sensitivity of the PHA assay.

In patients with pseudomonas infections, as well as in healthy control subjects, there was an excellent correlation between serum antitoxin titers determined by CTN and by PHA ($r =$

TABLE 3. Comparison of PHA and CTN antitoxin titers of sera from different animals immunized with purified *Pseudomonas aeruginosa* exotoxin

Animal	PHA titer (\log_2)	CTN titer (\log_2)
Rabbits		
Elfin	13	12
3N4	13	11
3N2	13	10
Sheep	18	15
Goat	17	16

0.91, $P < 0.001$), but the latter assay was between four and eight times as sensitive (Fig. 1).

The mean PHA titer, expressed as \log_2 of 9.4 ± 3.9 , of convalescent sera from 16 patients recovering from well-documented acute *pseudomonas* infections was significantly higher than the mean titer, 2.8 ± 2.6 , of sera from 17 healthy laboratory personnel ($P < 0.001$). As shown in Fig. 2, which depicts individual PHA assay results, there is a rather clear separation between the titers of patients postinfection and those of healthy controls. Eleven of seventeen control subjects, none of whom had a known history of prior *pseudomonas* infection, had at least some detectable antitoxin, although with two exceptions PHA titers were $< 1:32$. Likewise, among a small group of seven patients with serious bacterial, fungal, and viral infections, no convalescent PHA titers exceeded $1:16$, and no significant rises in titer were noted.

Five of seven patients with acute *pseudomonas* infections from whom paired sera were obtained demonstrated a fourfold or greater rise in PHA titer (Fig. 3). The two nonresponsive patients had underlying diseases (aplastic anemia and hairy-cell leukemia) that may have interfered with their ability to produce antibodies. The patient with the most rapid and dramatic rise in antitoxin titer, from an acute titer of $1:64$ to a convalescent titer of $1:128,000$ in less than 2 weeks, had had a well-documented bacteremic *pseudomonas* infection during a previous hospitalization and thus may have been manifesting an accelerated or anamnestic response.

Studies performed on acute and convalescent specimens from several patients revealed PHA antibody to be 2-ME-resistant and associated with the second (immunoglobulin G) protein peak obtained by Sephadex G-200 gel filtration chromatography of whole sera.

DISCUSSION

Our PHA assay for antibody to *P. aeruginosa* exotoxin is simple to perform, rapid, sensitive,

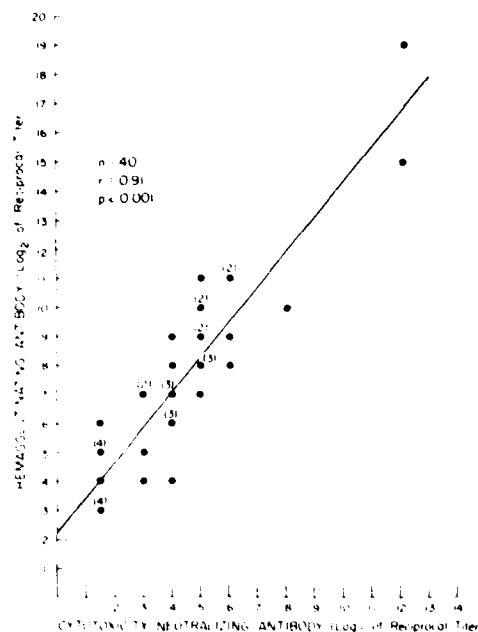


FIG. 1. Correlation between antitoxin titers obtained by PHA and by CTN assays performed on 40 serum samples from patients with *P. aeruginosa* infections and normal controls. The regression line shown was computed by the method of least squares.

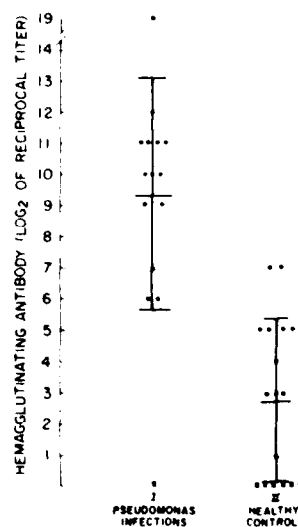


FIG. 2. Serum PHA antitoxin titers of 16 patients convalescing from *P. aeruginosa* infections compared with 17 healthy control subjects with no prior history of infection. Mean titers ± 1 standard deviation are indicated by brackets.

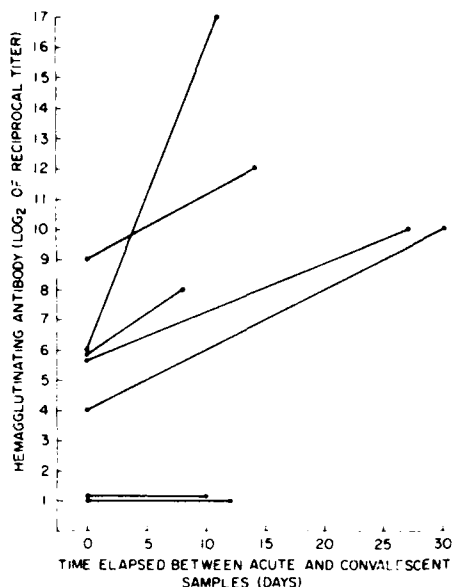


FIG. 3. Acute and convalescent serum PHA antitoxin titers of seven patients with acute *P. aeruginosa* infections.

and reproducible. Antitoxin titers obtained by this assay correlate well with those measured by our previously described CTN assay, and the newer method is between four and eight times as sensitive.

The demonstration of high serum PHA antitoxin titers in patients convalescing from pseudomonas infections, compared with healthy controls, and the significant titer rises found in some of the patients studied sequentially provide immunological evidence for the *in vivo* release of toxin during systemic infections in amounts sufficient to elicit antibodies. The lower antitoxin titers demonstrated in 11 of 17 healthy controls suggest acquisition of antitoxin by normal subjects, resulting from asymptomatic infection or colonization.

The survival of all five patients who showed PHA titer rises and the death of one of two patients who did not, as well as the demonstration of high antitoxin titers in most survivors of

pseudomonas infections, suggest a protective effect of serum antitoxin. The protection afforded pseudomonas-infected mice by the prior administration of antitoxin serum (O. Pavlovskis and M. Pollack, unpublished data) leads to a similar conclusion. Sequential PHA titers are now being determined in a larger group of patients with pseudomonas infections to establish this possible relationship between serum antitoxin levels and prognosis.

In addition to its usefulness in helping to delineate the possible pathogenic role of exotoxin and the protective effect of toxin specific antibodies, the measurement of pseudomonas antitoxin in human sera by the PHA assay may prove diagnostically useful. Supporting this conclusion are the ubiquity of exotoxin among, and its specificity for, *P. aeruginosa* strains (5), its apparent lack of important cross-reactivity with other bacterial antigens (5), the frequency and relative ease of measuring antitoxin responses in infected patients, and the rather clear separation between the higher antitoxin levels found in clinically infected patients and the lower or absent titers found in uninfected control subjects.

ACKNOWLEDGMENTS

We thank Lynn T. Callahan for supplying exotoxin, Richard Wistar, Jr., for his helpful advice, and Emilio Weiss and John B. Robbins for reviewing the manuscript.

LITERATURE CITED

1. Callahan, L. T. 1974. Purification and characterization of *Pseudomonas aeruginosa* exotoxin. *Infect. Immun.* 9:113-118.
2. Callahan, L. T. 1976. *Pseudomonas aeruginosa* exotoxin: purification by preparative polyacrylamide gel electrophoresis and the development of a highly specific antitoxin serum. *Infect. Immun.* 14:55-61.
3. Gold, E. R., and H. H. Fudenberg. 1967. Chromic chloride: a coupling reagent for passive hemagglutination reactions. *J. Immunol.* 99:859-866.
4. Pollack, M., L. T. Callahan, and N. S. Taylor. 1976. Neutralizing antibody to *Pseudomonas aeruginosa* exotoxin in human sera: evidence for *in vivo* toxin production during infections. *Infect. Immun.* 14:942-947.
5. Pollack, M., N. S. Taylor, and L. T. Callahan. 1977. Exotoxin production by clinical isolates of *Pseudomonas aeruginosa*. *Infect. Immun.* 15:776-780.
6. Vyas, G. N., H. H. Fudenberg, H. M. Pretty, and E. R. Gold. 1968. A new method for genetic typing of human immunoglobulins. *J. Immunol.* 100:274-279.