Application of FA Techniques to Detection of Clostridium perfringens

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FOOD POISONING caused by *Clostridium perfringens* (*welchii*) has, in recent years only, received appreciable attention. Many States, however, have never reported an outbreak of food poisoning caused by this organism.

Kemp and associates (*1*), in a review of the incidence of food poisoning in California, reported that approximately 50 percent of all food poisoning outbreaks were classified as "etiology unknown." They concluded that perhaps a considerable number of these may have been due to *C. perfringens*, which was overlooked because anaerobic cultures were not performed. On the national level, statistics are quite similar. In 1962, 214 food poisoning outbreaks were reported to the Public Health Service's Communicable Disease Center, and 111 of these were listed as "etiology unknown" (*Morbidity and Mortality Weekly Report*).

Historically, McClung (*2*) reported several outbreaks attributable to large numbers of *C. perfringens* organisms in foods containing chicken. More recently Hobbs and associates (*3*) reviewed the literature and made a rather complete study of the situation in England. These authors maintain that *C. perfringens* food poisoning is usually caused by heat-resistant, nonhemolytic strains of the organisms. In the United States, Hall and associates (*4*) studied various so-called food poisoning strains and concluded that any strain of type A *C. perfringens* can cause food poisoning, if present in adequate numbers.

Symptoms (*3*) of *C. perfringens* food poisoning are relatively mild; the victims usually experience gastrointestinal upsets, diarrhea, and cramps within 8 to 15 hours after eating food containing 10 million or more organisms per gram.

The species *C. perfringens* consists of a group of closely related strains of gram-positive, nonmotile, spore-forming anaerobic bacteria. They are widely distributed in nature, cause several different kinds of illnesses in men and in other animals, produce a variety of toxins, and are morphologically and biochemically similar to each other. The most practical method of differentiation, initiated by Wilsdon (*5*), is based on the pattern of toxin production exhibited by various strains. Subsequent investigators have systematized the major lethal toxin patterns, and currently five toxigenic types are recognized and are designated alphabetically from A to E (*6*). Type A most commonly affects man. Wilsdon also investigated the cellular antigens of *C. perfringens*, and he and subsequent investigators concluded that the species is antigenically diverse. Recently, Ellner and Bohan (*7*) studied the soluble antigens and concluded

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that the extreme heterogeneity of the somatic antigens apparently extends also to the soluble antigens.

Geck and Szántó (*8*) applied fluorescent antibody (FA) techniques to the study of *C. perfringens* and, although their studies were not extensive, they reported that *C. perfringens* conjugates were specific and produced brilliant fluorescence with homologous antigens.

This study was undertaken to obtain fundamental data on antigenic relationships of strains of *C. perfringens* with the hope that these would form a basis for their rapid identification by FA techniques. A specific serologic test of this kind not only would permit the diagnosis of *C. perfringens* food poisoning, but also might make it possible to type strains for epidemiologic tracing.

Materials and Methods

In this study 150 different strains of *C. perfringens* were obtained from a variety of sources, including those from clinical cases (14 different States), food-associated isolates, and university culture collections. Toxigenic strains from A to E were represented, although most cultures were type A. The immediate sources of these strains were: Robert A. Taft Sanitary Engineering Center, 47 cultures; Columbia University, 36; Montana State College, 11; Indiana University, 5; Central Public Health Laboratory (Food Hygiene) England, 13; Communicable Disease Center, 33; California Department of Public Health, 3; and miscellaneous isolates, 2.

When received, the cultures were checked for purity and correctness of identification by microscopic, biochemical, and toxigenic tests (*9*). Stock cultures were stored frozen in brain media and working cultures were maintained in cooked meat media.

Antigens were prepared by growing the organisms in modified dextrose broth, sedimenting by centrifugation, and washing thoroughly with 0.4 percent formalized distilled water followed by formalized saline. Turbidity was standardized by suspending the sedimented organisms in formalized buffered saline (pH 7.4) until the turbidity had a 50 to 65 percent transmittance on a Coleman Junior Spectrophotometer (λ 640 mμ). These antigens proved stable at refrigerator temperature for many months. Antiserums were produced in rabbits for 56 different strains of *C. perfringens*. The antiserums for the 13 Hobbs' food poisoning strains were prepared by the method of Henderson (*10*) and for the remainder by the method of Hall and associates (*4*).

Both of these procedures essentially consisted of injecting rabbits intravenously with a series of increasing doses of antigen. Blood specimens were drawn from the rabbits about 1 week after the last injection. Serums were separated, merthiolate was added (final concentration 1:5,000), and the serums were stored frozen. A portion of each antiserum was conjugated with fluorescein isothiocyanate (FITC) for fluorescent antibody (FA) stains. The basic method was that of Riggs and associates (*11*). Slight modifications were instituted, such as dissolving the FITC in the carbonate-bicarbonate buffer before adding it to the globulin solution and removing free fluorescein after conjugation with alkalinized Dowex (Dowex rinsed with pH 8.4 saline and drained). Conjugates were preserved with merthiolate in a final concentration of 1:5,000 and stored frozen.

Antiserums had macroscopic slide agglutinating titers ranging from 1:80 to 1:320 and the conjugates proved satisfactory at dilutions of 1:25 to 1:100.

Results

For convenience of manipulation and study, the 56 antiserums and their homologous antigens and conjugates were divided into five separate groups. The first group consisted of the 13 Hobbs' (English) food poisoning strains. Each of the other four groups contained from 7 to 14 different antigenic strains of the organism. To study the antigenic and serologic relationships of the different strains of the organisms, slide agglutination tests and FA staining were performed on homologous and heterologous antigens. Typical results are shown in table 1 (13 Hobbs' strains), which demonstrates the extreme antigenic strain specificity of the organisms. Antiserum and conjugate No. 5 had a low staining titer and crossed with several heterologous antigens. Subsequently, more specific antiserum was produced for this strain.

Table 1. Comparative results of slide agglutination and fluorescent antibody reactions using the Hobbs' food poisoning strains of Clostridium perfringens (type A)[1]

Antigen	Tests	Antiserums and conjugates												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Agg	320	N	N	N	N	N	N	N	N	N	N	N	N
	FA	100	N	N	N	2–3+	N	N	N	N	N	N	N	N
2	Agg	N	160	N	N	N	N	N	N	N	N	N	N	N
	FA	N	100	N	N	N	N	N	N	N	N	N	N	N
3	Agg	N	N	320	N	N	N	N	N	N	N	N	N	N
	FA	N	N	100	2+	N	N	N	N	N	N	N	N	N
4	Agg	N	N	N	80	N	N	N	N	N	N	N	N	N
	FA	N	N	2–3+	100	2–3+	N	N	N	N	N	N	N	N
5	Agg	N	N	N	N	320	N	N	N	N	N	N	N	N
	FA	N	N	N	N	<25	N	N	2–3+	N	N	N	N	N
6	Agg	N	N	N	N	N	80	N	N	N	N	N	N	N
	FA	N	N	N	N	N	100	N	N	N	N	N	N	N
7	Agg	N	N	N	N	N	N	160	N	N	N	N	N	N
	FA	N	N	N	N	N	N	100	N	N	N	N	N	N
8	Agg	N	N	N	N	N	N	N	320	N	N	N	N	N
	FA	N	N	N	N	2–3+	N	N	50	N	N	N	N	N
9	Agg	N	N	N	N	N	N	N	N	160	N	N	N	N
	FA	N	N	N	N	N	N	N	N	50	N	N	N	N
10	Agg	N	N	N	N	N	N	N	N	N	320	N	N	N
	FA	N	N	N	N	N	N	N	N	N	100	N	N	N
11	Agg	N	N	N	N	N	N	N	N	N	N	320	N	N
	FA	N	N	N	N	N	N	N	N	N	N	100	N	N
12	Agg	N	N	N	N	N	N	N	N	N	N	N	160	N
	FA	N	N	N	N	2+	N	2+	N	N	N	N	100	N
13	Agg	N	N	N	N	N	N	N	N	N	N	N	N	320
	FA	N	N	N	N	N	N	N	N	N	N	N	N	50

[1] Pool No. 1: See text for explanation.

NOTE: Titers are given for homologous strains only. The end point was read at 4+ for both agglutination (Agg) and fluorescent antibody (FA) reactions. The titers shown are reciprocals of the dilutions. Dilutions were made no greater than 1:320 for agglutinations and 1:100 for FA. Results on heterologous strains were determined at diagnostic levels; that is, agglutination tests were performed at a 1:60 dilution of serum (recorded + or N, negative), and FA readings at a 1:25 to 1:50 dilution of conjugate, depending on its titer. Readings of less than 2+ recorded as negative, N.

Studies with the four other antigen-antiserum-conjugate groups gave similar results and confirmed the general pattern of antigenic strain specificity. To avoid repetitious production of serums from similar or identical strains, each antigen, before selection for use in immunizing rabbits, was screened with the antiserums from all preceding groups by means of slide agglutination tests. Group No. 5 consisted of toxigenic strains of types B, 5 strains; C, 5; D, 6; E, 3; and F, 3; a total of 22 strains whose toxin types were determined during this study by employing neutralization tests in mice. In this rather limited study all the type B strains appeared to be related antigenically, but all other toxigenic types were antigenically diverse.

In general, smears stained with homologous conjugates fluoresced brilliantly. The pattern of FA staining and quellung reactions with antiserum indicated that the antibodies were primarily directed toward the capsules of the organisms. These capsular antigens were stable to heat, dilute alkalies, and formalin, but were sensitive to hot dilute hydrochloric acid ($N/_{10}$).

A practical development was the finding that conjugates and antiserums could be satisfactorily pooled and strains of *C. perfringens* homologous to each pool could be easily selected and identified. Five different pools of both antiserums and conjugates were prepared by mixing the individual antiserums or conjugates within each of the five previously mentioned groups. For example, pool 1 contained antibody for each of the 13 Hobbs' strains of *C. perfringens*; pool 1 of the conjugates, likewise,

Table 2. Attempts to identify an assorted group of Clostridium perfringens cultures by the use of 5 pools and by individual antiserums and conjugates

Antigen	Tests	Antiserums and conjugates					
		Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Similarity [1]
Cal-946	Agg	+	N	N	N	N	Hobbs' 1.
	FA	4+	N	N	N	N	
S-95	Agg	+	N	N	N	N	Hobbs' 10.
	FA	4+	N	N	N	N	
A-129	Agg	+	N	N	N	N	Hobbs' 4.
	FA	4+	N	N	N	2+	
364	Agg	N	N	+	N	N	3,502.
	FA	N	N	4+	N	N	
R-2	Agg	N	N	+	N	N	3,502.
	FA	N	N	4+	N	N	
R-3	Agg	wk+	N	N	N	N	Hobbs' 11.
	FA	4+	N	N	N	2+	
196-A	Agg	+	N	N	N	N	Hobbs' 11.
	FA	4+	N	N	N	N	
153	Agg	wk+	N	N	N	N	Hobbs' 11.
	FA	3+	N	N	2	N	
21 (type A)	Agg	N	N	N	N	+	462 (type D).
	FA	N	N	N	N	4+	
103	Agg	N	N	+	N	N	109-A.
	FA	N	N	3+	N	N	
209-A	Agg	+	N	N	N	N	Hobbs' 3.
	FA	4+	N	N	N	N	
216-A	Agg	N	+	N	N	N	Cal 1,072.
	FA	N	3-4+	N	2+	N	
1-Eln	Agg	N	+	N	N	N	IU-690.
	FA	N	4+	N	N	N	
IU-2114	Agg	+	N	N	N	N	Hobbs' 6.
	FA	4+	N	N	N	N	
IU-1333	Agg	+	N	N	N	N	Hobbs' 2.
	FA	4+	N	N	N	N	
B-14	Agg	+	N	N	N	N	Hobbs' 4.
	FA	4+	N	N	N	N	

[1] Determined by screening the unknown antigen with each of the 5 pools of antibodies. When a positive result was obtained, specific identification was made by examining the antigen with each constituted antibody making up the pool. Agglutination (Agg) tests were performed at 1:60 (recorded as + or N, negative), and fluorescent antibody (FA) readings at 1:25 or 1:50 dilutions of the conjugate, depending on the titer of the individual conjugates making up the pools. Readings of less than 2+ are recorded as negative, N.

contained labeled antibody for each of these 13 strains (table 1). The amount of each component used in the pool was adjusted in accordance with the diagnostic titer of the antiserum or conjugate. Staining and agglutination results showed little crossing between pools.

Having established five separate and rather specific antiserum and conjugate pools, we decided to determine how many strains of *C. perfringens* (not used in the preparation of antiserum) could be identified by testing with the pools of reagents. In this way, unknown cultures of *C. perfringens* could be screened for serologic relationship to the strains used for preparation of the pools. Reacting strains then could be identified specifically by testing with individual antiserums and conjugates within the pool. A group of 79 cultures of *C. perfringens* were used in this study.

The practical use of the five antibody and conjugate pools is demonstrated and typical reactions are shown in table 2. Of the 79 strains tested, 34 gave strongly positive results with one of the antiserum and conjugate pools. Each was subsequently identified as being identical or closely related to one of the individual antigens used to produce the antiserums. Hobbs' strains were observed 21 times in these antigenic similarities. The frequency and distribution of types was: type 8, 5 times; type 4, 4; type 11, 4; type 5, 2; type 10, 2; and types 1, 2, 3, and 6, one time each. Another interest-

Table 2. Attempts to identify an assorted group of Clostridium perfringens cultures by the use of 5 pools and by individual antiserums and conjugates—Continued

Antigen	Tests	Antiserums and conjugates					
		Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Similarity [1]
224–T	Agg	+	N	N	N	N	Hobbs' 4.
	FA	4+	N	N	N	N	
227–T	Agg	N	N	+	N	N	109–A.
	FA	N	N	3+	2+	N	
F–238–T	Agg	+	N	N	N	N	Hobbs' 5.
	FA	4+	N	N	2+	2+	
F–236–T	Agg	+	N	N	N	N	Hobbs' 8.
	FA	4+	N	N	N	N	
F–244–T	Agg	+	N	N	N	N	Hobbs' 10.
	FA	4+	N	N	N	N	
229–B	Agg	N	N	N	+	N	228–A.
	FA	N	N	N	4+	N	
212–T (type A)	Agg	N	N	N	N	+	1,493 (type E).
	FA	N	N	N	N	4+	
211–T (type A)	Agg	N	N	N	N	+	12 (type C).
	FA	N	N	N	N	4+	
209–T	Agg	+	N	N	N	N	Hobbs' 8.
	FA	4+	N	N	N	N	
206–T	Agg	+	N	N	N	N	Hobbs' 8.
	FA	4+	N	N	N	N	
138–T	Agg	+	N	N	N	N	Hobbs' 4.
	FA	+	N	N	N	N	
S–141–T	Agg	+	N	N	N	N	Hobbs' 5.
	FA	4+	N	N	2+	N	
F–254–T	Agg	+	N	N	N	N	Hobbs' 8.
	FA	4+	N	N	N	N	
123–T	Agg	N	+	N	N	N	IU–2,825.
	FA	N	4+	N	N	N	
140–T	Agg	+	N	N	N	N	Hobbs' 11.
	FA	4+	N	N	N	N	
163–T	Agg	+	N	N	N	N	Hobbs' 8.
	FA	4+	N	N	2+	N	
114–T	Agg	N	N	+	N	N	604.
	FA	N	N	4+	N	N	
19	Agg	N	N	+	N	N	3,502.
	FA	N	N	3–4+	N	N	

ing feature was that toxigenic types C, D, and E were occasionally serologically related to type A strains (table 2).

In addition to the 34 strains serologically identified, 18 others were completely negative and 27 gave equivocal results. From the data in table 2 it is clear that the results of slide agglutination tests closely parallel those of FA staining, although the latter generally were the more sensitive indicators of antigen similarities. One advantage of the FA test was that the serologic characteristics of *C. perfringens* could be determined without purification of the culture or specimen.

Since antiserum and conjugate pools appeared to be useful for the rapid screening of unknown cultures, we decided to explore the possibility of producing polyvalent antiserum in a single rabbit. An antigen of five times the usual concentration was prepared, which contained equal numbers of all the 13 Hobbs' strains of *C. perfringens*. Five rabbits were immunized in the usual manner and blood specimens were drawn a week after the last injection. We noted that antibodies in satisfactory titer were developed by all the rabbits to most of the strains. One rabbit produced substantial titers of both agglutinating and FA antibodies to all 13 strains.

The specificity of *C. perfringens* antiserums and conjugates was evaluated by testing them against 15 environmentally associated species of aerobes and anaerobes from the Communicable Disease Center culture collections, including

Bacillus cereus, 5 strains; *Bacillus subtilis*, 3; *Clostridium bifermentans*, 3; *Clostridium sordelli*, 2; *Clostridium multifermentans*, 3; *Clostridium botulinum* types A and B, 2 each; *Clostridium subterminale*, 2; *Clostridium paraputrificum*, 1; *Clostridium septicum*, 3; *Clostridium histolyticum*, 3; *Clostridium butyricum*, 1; and *Clostridium tetani*, 1. There were no cross agglutinations and the only serious cross staining was with *Clostridium septicum*. Studies showed that these cross staining antibodies could be easily absorbed without apparent diminution of the staining intensity for homologous *C. perfringens* antigens.

Hobbs' strains of *C. perfringens* were experimentally cultivated in a number of different kinds of food, including tuna loaf, shrimp creole, chili beans, beef stew, chicken a la king, and a variety of meat soups. In every instance capsules were produced at temperatures ranging from 25° C. to 46° C. Smears were made directly from the food, defatted, fixed with acetone, and stained with homologous conjugate pools. The *C. perfringens* organisms present fluoresced brilliantly and there was little background fluorescence. A further practical point was that food containing *C. perfringens* could be preserved with formalin (up to 5.0 percent) and held for at least 2 months at 37° C. without impairing the staining characteristics of the organisms.

Twenty-one human fecal specimens were stained with *C. perfringens* conjugate pools. Even though homologous organisms were known to be present, they stained poorly or not at all. The assumption was that satisfactory capsules were lacking. When a sample of feces was cultured first in dextrose cooked meat broth, then exposed to homologous conjugates, the organisms stained brilliantly. A matter of practical importance was that pooled conjugates stained some strains of enterococci in direct fecal smears. Although these fluoresced brilliantly, they were readily differentiated by morphology.

Discussion

This preliminary research points up some fundamental possibilities and problems. Antigenically, *C. perfringens* is highly strain specific and no one knows how many strains there are. Their great diversity is shown by the fact that only 34 strains could be serologically identified by the use of 56 conjugates and antiserums, from a total of 79 unknown strains of *C. perfringens*. On the other hand, Dr. Betty C. Hobbs (personal communication, 1964) stated that, in England, her 13 types had caused more than 70 percent of all reported *C. perfringens* food poisoning. Except for the work of Hall and associates (*4*), little is known of the serology of food poisoning strains of *C. perfringens* in the United States. However, our study indicated that the Hobbs' strains are quite prevalent in this country. Accordingly, they can be expected to be encountered frequently in connection with outbreaks of food poisoning.

C. perfringens conjugates are stable; pooled conjugates gave good results, and homologous organisms fluoresced brilliantly. FA staining techniques appear to be a practical tool for the preliminary detection and identification of encapsulated strains of *C. perfringens*. Subsequent research may result in the development of other pools that will assist in the identification of most strains of the organism. Smears from foods suspected of causing food poisoning are easily prepared, fixed, defatted with acetone, and stained; even from specimens preserved with formalin. Using FA procedures, presumptive results can be available in an hour or two. Since food specimens can be preserved with formalin, they may be satisfactorily mailed without refrigeration. Also, FA techniques offer the possibility of epidemiologically associating the strain of *C. perfringens* present in food with the strains present in the patient's feces. Even though the feces must first be cultured before smears are made, there is no need for time-consuming colony isolation.

In a thorough study of any food poisoning outbreak it is important to estimate accurately the number of organisms present. By the use of carefully sampled food, accurate dilutions, and measured aliquots for the preparation of smears, it is entirely possible to make rapid and rather accurate estimates of bacterial numbers in FA-stained preparations. Conventional bacteriological procedures also can be used (*12*).

Since cells of *C. perfringens* in food samples

stain well, we can anticipate that smears made from suspected gas gangrene exudates or from tissue sections or imprints will stain well if homologous oganisms are present. If this is the case, FA techniques should be a valuable tool for early presumptive diagnosis of this disease.

Summary

One hundred and fifty strains of *Clostridium perfringens* (toxigenic types A to E) were obtained from a variety of sources. The identity of these cultures was confirmed by standard bacteriological techniques. Employing formalin-treated antigens, serums were prepared in rabbits for 56 of these strains. The serums and the fluorescein labeled conjugates derived from them were arranged into five pools for screening cultures by slide agglutination and fluorescent antibody (FA) tests, respectively. These reagents were shown to be highly specific for the capsular antigen when the capsular swelling and FA tests were employed. The capsular antigens proved stable to heat, dilute alkali, and formalin, but were sensitive to hydrochloric acid.

The five pools of FA reagents were used in an attempt to type a group of 79 unknown cultures of *C. perfringens*. Thirty-four of these were typable, and 21 were identified as Hobbs' serotypes. With one exception, antiserums and conjugates were found to be specific for *C. perfringens* when tested against cultures of 10 other species of *Clostridia* and 4 species of *Bacillus*.

C. perfringens was experimentally grown in a variety of foods and the encapsulated organisms were brilliantly stained by the appropriate FA reagent in smears made directly from the food. Tests showed that food samples could be preserved with 1.0–5.0 percent formalin for 2 months or longer without impairing the FA staining characteristics.

Mixed strains of *C. perfringens* were commonly found in human feces, but the organisms apparently were not well encapsulated. Their presence was easily demonstrated by a combination of enrichment and FA techniques without the necessity of obtaining pure cultures.

It was concluded that immunofluorescence may prove to be a valuable tool for the rapid identification and enumeration of *C. perfringens* in food and feces during investigations of food poisoning outbreaks.

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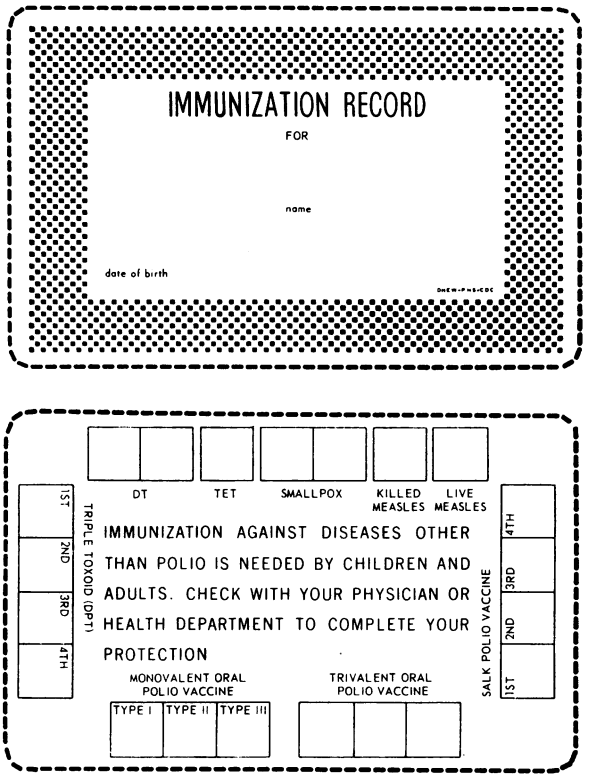
Wallet-Size Immunization Record Cards

In March 1964, the Communicable Disease Center, Public Health Service, in Atlanta, Ga., undertook a study to: (*a*) provide quick and accurate means of recording oral, mass immunizations; (*b*) motivate individuals to seek additional, needed immunizations; and (*c*) provide permanent, wallet-sized record cards. A plastic card, punched to show that the person had received his first dose of oral vaccine, was given to every person who attended the first Sabin Oral Sunday in Columbus, Ga. More than 50,000 cards were distributed at 50 community sponsored polio vaccine clinics, with a reminder to bring the card back for the second round.

At the five clinics used as sampling stations, 92.1 percent of the 6,606 persons interviewed brought the cards to the second immunization to be punched. All persons returning without the card were given a second one and everyone was reminded to bring the card to the third round in May. During the final Sunday in May, 5,830 or 95.3 percent of 6,115 persons at eight clinics returned with cards to be punched. The high rate of return applied to all economic levels; in fact, lower socioeconomic groups had a return rate about 5 percent better than average.

At the last round everyone was given a leaflet explaining the purpose of the card and urging that it be retained and taken to the family physician or health department whenever an immunization is received. The leaflet also pointed out that children, with a few exceptions, should be immunized before the first birthday against DTP, poliomyelitis, and smallpox. Adults should receive protection against smallpox and tetanus every 3 to 5 years and complete poliomyelitis immunization.

The Columbus test strongly indicates the feasibility of using a plastic wallet-sized immunization record card. Another survey will be conducted in the spring of 1965 to determine how many people still have the cards and to what degree the cards are being used to maintain a complete record of immunization. No space for recording dates was a long term drawback of the punchcard used in Columbus. However, similar plastic cards can be devised to overcome this problem. Given the desired design, plastics companies can produce cards which have any number and size of roughened spaces which allow permanent writing with pen or pencil.



Both sides of record card