Can preserved erythrocytes be used for routine diagnostic testing? This study endeavors to answer the question. According to the authors, tests show that this is quite possible, and they recommend further trials in routine laboratory situations.

A STUDY ON THE DETECTION AND SPECIFICITY OF ANTIBODIES TO SHIGELLA FLEXNERI TYPES USING PRESERVED POLYSACCHARIDE-SENSITIZED HUMAN ERYTHROCYTES

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SINCE the introduction by Flick[1] in 1948, of preserved erythrocytes for the study of influenza A virus hemagglutinating activity, various modifications and applications have appeared in the literature. Cole and Farrell[2] overcame the spontaneous agglutination of the formalinized red blood cells encountered by Flick by dispersing the cells in a Waring blendor for 60 minutes. Protein antigens were then coupled to the preserved cells by the use of tetrazotized benzidine. Later McKenna[3] treated the preserved erythrocytes of Flick[1] with tannic acid to enable them to absorb bovine serum albumin and bovine gamma globulin. He still encountered some spontaneous agglutination. Cox[4] modified the formalin method to enable the use of preserved sheep erythrocytes for the titration of heterophile antibodies in infectious mononucleosis. Feeley, et al.[5] adopted the preservation method of Cox,[4] which eliminated much of the hemolysis and spontaneous agglutination, to prepare red blood cells for the enterobacterial hemagglutination test. The cells were sensitized with pathogenic Escherichia coli, Shigella flexneri, and Salmonella antigens, and remained stable for several months. Sieburth[6] used the same method for stabilizing standardized sensitized chicken erythrocytes for the Salmonella indirect hemagglutination test.

The advantages of stable erythrocytes which can be used over a long period of time, either readily available for sensitizing with fresh antigen preparations, or previously sensitized with one or more antigens for routine serum testing, are obvious. The present study was undertaken to determine the applicability of this technic to routine use in clinical laboratories for the detection of a rise in antibody titer in cases of bacillary dysentery.

Method

Essentially the method used for preparation of the preserved human erythrocytes (PHE) was the same as that employed by Feeley, et al.,[5] except that

triethanolamine buffered saline (TBS)[7] was substituted for phosphate buffered saline. The technic consists of the following: human, outdated group O Rh negative blood in acid citrate dextrose solution as preserved by the Red Cross was diluted in half with TBS which had been filtered for sterility. To 100 ml of the resulting diluted cells was added 40 ml of formaldehyde which had been made isotonic with an equal quantity of twice concentrated TBS. The cell suspension was then incubated for two hours in a 37° C water bath and swirled every 15 minutes to insure thorough mixing. The erythrocytes turned brown during this period. The browned erythrocytes were centrifuged, washed four times with TBS, resuspended, sedimented, and decanted over three 48 hour intervals. A 10 per cent suspension was then made in TBS to which 0.3 per cent formalin had been added as a preservative. This suspension was kept at 4° C throughout the period of study.

Preparation of the polysaccharide extracts used to sensitize the PHE has been described elsewhere.[8] Briefly, this consisted of heating smooth, agar grown saline suspensions of the bacteria together with an equal quantity of 0.1 N NaOH at 100° C for 70 minutes. The material was then cooled, adjusted to pH 7.0, dialyzed, centrifuged, and the supernate Seitz filtered. The extract was carefully standardized with hyperimmune rabbit antiserum to determine the lowest concentration giving a high stable titer.

Single polysaccharide sensitized cells were prepared in the following manner. The formalinized red cell suspension was washed three times in TBS and resuspended to make a 10 per cent suspension. This suspension was then added to an equal amount of polysaccharide extract which had already been diluted to its optimum reactive capacity as determined by standardization. The mixture was placed in a 37° C water bath for a two hour sensitization period, the cells washed again three times in TBS and resuspended to make a 1 per cent cell suspension in the same buffer to which 0.3 per cent formalin had been added. This stock of preserved sensitized human erythrocytes (PSHE) was stored at 4° C and used without further alterations or washing for the six month testing period.

PHE were also simultaneously sensitized[9] with polysaccharide antigens by exposing the washed 10 per cent formalinized cell suspension to an equal quantity of five different polysaccharide extracts. The polysaccharide solutions were prepared so that each was in its appropriate concentration for optimum sensitization.

Fresh human erythrocytes (FHE) were sensitized in the same way as the formalinized cells except that the fresh blood was preserved in Alsever's solution[10] and no formalin was added throughout the process.

Hemagglutination (HA) tests with both FHE and PHE were carried out in serial (twofold) dilutions of the hyperimmune rabbit antiserums and patient's serums. Equal amounts of cell suspensions and diluted serums were incubated for two hours in the 37° C water bath. The readings to determine serum antibodies, however, were carried out differently for the two types of cells. In tests performed with fresh cells the pattern formed by the clumped cells was noted, but the tubes were then tapped lightly and only those tubes showing complete agglutination of all the red blood cells was regarded as positive. The cells preserved by formalin do not have sufficient binding power to retain their adherence when tapped, so these tests were read by pattern only. Parallel readings made of the fresh blood cell tests to compare titer end points read by pattern and by complete clumping of the cells showed but little variation, so this latter procedure which had long been

Table 1—Six-Month Evaluation of Single and Polyvalent Sensitized Preserved Human Erythrocytes

Antiserum	Shigella flexneri HA Reactions									
	1a	1a	1b	1b	4a	4a	4b	4b	5	5
Polysaccharide antigen	1a	Pv*	1b	Pv	4a	Pv	4b	Pv	5	Pv
January	20,480†	10,240	2,560	2,560	10,240	2,560	2,560	2,560	5,120	5,120
February	10,240	5,120	1,280	1,280	2,560	1,280	1,280	1,280	5,120	2,560
April	10,240	5,120	2,560	2,560	2,560	2,560	2,560	640	10,240	10,240
May	10,240	10,240	2,560	2,560	10,240	10,240	2,560	1,280	5,120	5,120
June	10,240	10,240	5,120	2,560	5,120	5,120	1,280	640	5,120	5,120

* Pv = polyvalent.
† Reciprocal of the highest serum dilution yielding a definitive pattern.

in use in our laboratory was retained. Also, parallel tests had been set up to determine the optimum incubation time and temperature for the HA test using PHE and Shigella polysaccharide extracts. Feeley, et al.,[5] recommended that the HA tests be incubated for 30 minutes at room temperature. Readings of our tests at 30 minutes gave end points ranging from identical to three tubes lower than those at two hours. Furthermore, the cells had not all settled by the 30 minute period, thereby rendering the reading by pattern more difficult. Temperature seemed even more critical than time. A very few of the titers obtained by incubation at room temperature were the same as those obtained by incubation at 37° C, but most were from two to six tubes lower. Optimum conditions for the present HA studies were, therefore, incubation for two hours at 37° C. It should be noted that it is unnecessary to inactivate serums that will be tested with PHE since such cells are not subject to hemolysis.

Results and Discussion

The methods described above were employed to determine HA titers obtained with single polysaccharide sensitized preserved cells as compared with titers obtained when such cells were simultaneously sensitized with polysaccharide antigens prepared from Sh. flexneri 1a, 1b, 4a, and 5. Both single and polyvalent sensitized cells were tested for stability for a period of six months. Table 1 shows the results of the six month evaluation using hyperimmune rabbit antiserum for the testing. It can be seen that while slight variations occur there are no real differences in titer between tests run with single and polyvalent sensitized cells. Also the titers obtained in January and June usually are identical or show a one tube variation. In only one instance was a two tube difference seen. Flick[1] found that erythrocytes preserved by his method of formalin treatment were useful for a period of two years. It is probable that the PSHE prepared according to Feeley's method[5] could also be used for considerably longer than six months.

After the stability of the preserved erythrocytes was established, the next step was to determine their utility in the serology of actual cases of bacillary dysentery. Serums from an epidemic of Sh. flexneri 6 provided the opportunity for such an evaluation. The results are recorded in Table 2. Comparative

agglutination and HA tests had been carried out previously on these serums. In those instances where sufficient serums still remained for testing, the cells which had been sensitized six months before with polysaccharide extracts from Sh. flexneri 6 were used for HA tests. As previously reported, the agglutination tests gave very poor results while the HA tests gave high titers.[11] No real difference could be detected between the original fresh erythrocyte HA test and the HA test using preserved erythrocytes six months old.

To further verify the results obtained with the Sh. flexneri 6 patients, five serums from children with sporadic infection of Sh. flexneri 3 were tested. These are shown in Table 3. The observed variation in titer is minimal with the exception of the acute phase serum of patient No. 2. This shows that rare differences can occur.

While much is known about the common constitutents of some of the Sh. flexneri polysaccharides,[12] and while the cross agglutination reactions of these strains in hyperimmune antiserums are well established,[13] the homologous and heterologous hemagglutinin response to Sh. flexneri infections in patients remained to be determined. Such studies were carried out on serums from a Sh. flexneri 6 patient, using HA antigens prepared from Sh. flexneri types 1b, 2a, 3, and 4a, all of which are reported to give cross-agglutination reactions.[13] Antibody response to the homologous strain Sh. flexneri was also tested. The results are given in Table 4. The only one of the strains which did not show a rise in antibody titer is Sh. flexneri 2a which retained a titer of 32. Sh. flexneri types 3 and 4a both showed a two tube rise in hemagglutinin titer, the 1b a three tube rise. The homologous Sh. flexneri 6 strain stimulated a four tube rise which was not a great deal higher than the others, but does reach a significant level. It is obvious that the specificity of the HA test for strains within the Sh. flexneri group is not

Table 2—Serological Studies of Patient Serums Obtained from an Epidemic of Shigella flexneri 6

Pt. No.	Acute Serum Titers			Convalescent Serum Titers		
	Agglutination	FSHE	PSHE	Agglutination	FSHE	PSHE
1	—	128	64	16	256	256
2	0	0	0	0	256	256
				0*	128*	128*
3	0	0	0	—	—	—
4	0	64	64	—	—	—
5	0	1,024	2,048	64	1,024	512
6	0	32	32	0	128	128
7	0	1,024	1,024	16	1,024	—
8	0	128	256	8	512	1,024
9	0	0	0	0	128	128
10	0	0	—	0	1,024	1,024
11	—	32	128	0	64	128

—=Test not done; serum not available.
FSHE=Fresh sensitized human erythrocytes.
PSHE=Preserved sensitized human erythrocytes.
* Second convalescent serum drawn on patient No. 2.

Table 3—Serological Studies of Patient Serums Obtained from Sporadic Cases of Shigella flexneri 3*

Patient No.	Serum	Hemagglutination Titers FSHE	PSHE
1	Convalescent	512	128
2	Acute	32	256
	Convalescent†	256	256
3	Acute	16	32
	Convalescent	256	256

* Obtained from Children's Hospital, Washington, D. C.
† Convalescent serums were drawn ten days after the acute serums.
FSHE = Fresh sensitized human erythrocytes.
PSHE = Preserved sensitized human erythrocytes.

Table 4—Homologous and Heterologous Hemagglutinin Response to Shigella flexneri Types in a Patient with Shigella flexneri 6 Infection

Types of Shigella flexneri HA Antigen	Hemagglutination Titers Acute Serum (September 12)	Convalescent Serum (September 22)
1b	128	1,024
2a	32	32
3	64	256
4a	256	1,024
6	64	1,024

strong. This may be explained by the identical polysaccharide constitutents among the Sh. flexneri serotypes.[12]

As previously stated, to establish end points of the HA titer when fresh red blood cells are used, only complete agglutination of all the cells is regarded as positive. The PHE tests, on the other hand, are read by pattern. Figure 1 presents parallel tests using the two types of cells in which the first two tubes are positive; the third tube of each is a ± reading (considered a negative reaction). The fresh erythrocytes gave a positive pattern in this third tube, but agitation revealed incomplete clumping. The last two tubes of each series are negative. It is apparent from this illustration that more experience is required to read the tests with preserved cells accurately. The next three illustrations (Figures 2, 3, and 4) are shown to depict more clearly those features on which the end point reading is based in the PSHE hemagglutination test. Figure 2 shows a positive test and corresponds to the second tube in the preceding illustration. The next one (Figure 3) shows a ± reaction with its early stage in the formation of the typical negative "doughnut." Figure 4 shows the fully developed negative "doughnut" ring. It should be mentioned that patient serums tend to give less easily read end points than the hyperimmune rabbit antiserums, but with experience in the use of this method the results are reproducible.

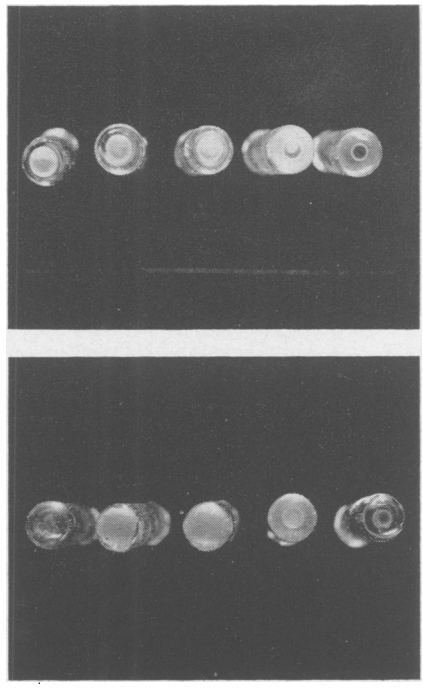

Figure 1—Hemagglutination Tests with: A. Fresh Human Erythrocytes (FHE) B. Preserved Human Erythrocytes (PHE)

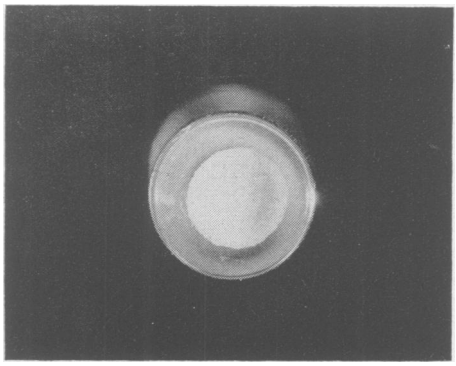

Figure 2—Hemagglutination with Preserved Human Erythrocytes: Positive Reaction

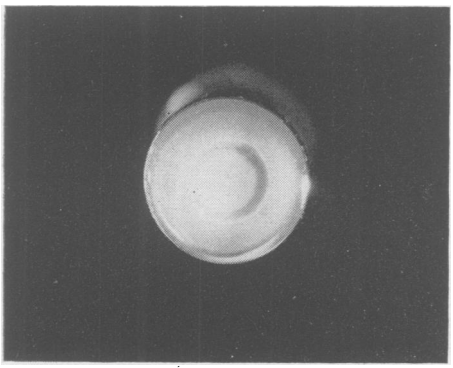

Figure 3—Hemagglutination with Preserved Human Erythrocytes: Plus-Minus Reactions (Read as Negative)

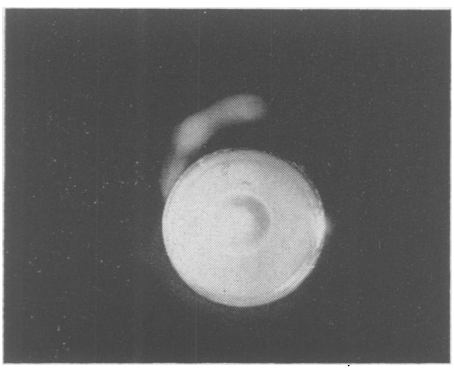

Figure 4—Hemagglutination with Preserved Human Erythrocytes: Negative Reaction

The enterobacterial hemagglutination test has been established as affording an accurate and sensitive test for the detection of antibodies. For example, in the outbreak of Shigellosis reported by Sochard, et al.[11] not a single case would have been diagnosed on the basis of agglutinin response, whereas every individual showed a prompt rise in hemagglutinins to a high titer. Neter[14] has reviewed this subject in detail. With the advent of formalin preserved cells new vistas in the utilization of the HA test have opened. PHE afford a simple, lasting source of cells wherever the supply would be difficult to obtain. They will last for many months in a sensitized or unsensitized state. The potential for their use in smaller or field laboratories is very large. It would be possible for centrally located laboratories to prepare large volumes of preserved sensitized carefully standardized cells for small or field laboratories. In consequence, the results over long periods of time would become more uniform. The smaller laboratories would simply have to add the proper serum dilutions without inactivation to run an effective test. We strongly recommend that this test be given extensive trials under the conditions stated.

Summary

Studies were presented in which formalinized erythrocytes (PHE) sensitized with single or multiple polysaccharide antigens were tested for stability over a period of six months. No real differences or diminution of titer could be detected. Comparative HA tests with fresh and preserved cells carried out on serums from patients with bacillary dysentery gave the same results. The sensitivity and stability of this test, together with the obvious advantages it affords, make it worthy of extensive trial for widespread use as a routine diagnostic test.

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This paper was presented before the Laboratory Section of the American Public Health Association at the Eighty-Seventh Annual Meeting in Atlantic City, N. J., October 21, 1959.

Public Health Is Where People Are

In August, the Tuberculosis Control Service of the San Diego Department of Public Health installed an x-ray machine in the San Diego County Jail to screen all new prisoners for unsuspected active tuberculosis.

In the first 27 days of operation, 1,253 x-rays were taken. Three persons who were unaware that they had the disease were found to have tuberculosis: One had a minimal case; one was moderately advanced; one was far advanced. All three prisoners are now hospitalized and are receiving medical treatment.

The health department expects that "every 400 to 500 jail x-rays will find a person with tuberculosis. Normally, a case is found in our population at large in every 5,000 to 8,000 chest x-rays."