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The Use of Fixed, Sensitized, Lyophilized Erythrocytes for the Middlebrook-Dubos Tuberculin Test.* (27135)

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The purpose of this paper is to describe the use of fixed sensitized sheep erythrocytes in the Middlebrook-Dubos hemagglutination test for tuberculosis. The classical test described by Middlebrook and Dubos(1), and the modified test by Scott and Smith(2), and others(3,4) involves the use of fresh unfixed erythrocytes. As such, the preparation for the individual tests is rather time-consuming. A diagnostic reagent which could be directly used would be of great advantage.

With a view toward development of a rapid, convenient screening test for toxoplasmosis, the author has previously described a stable preparation of alcohol-formalin fixed, Toxoplasma-sensitized lyophilized sheep erythrocytes(6). These antigen coated cells could be stored succesfully for a period of at least 2 months. Encouraged by these results the author has attempted to adapt this fixation technic to the modified Middlebrook and Dubos test.

Material and methods. 1. *Sheep erythrocytes.* Sheep blood collected aseptically in 1.2 volumes of modified Alsever's solution(1) was kept for at least 3 days before use, and was used thereafter for a period up to 2 weeks. Before fixation, the cells were washed 3 times with normal saline by serial centrifugation. When the fresh washed cells were used in the test, the final suspensions were centrifuged at 2,000 rpm for 10 minutes.

2. *Fixation of washed cells.* One volume of washed packed cells was suspended in 9 volumes of isotonic phosphate-buffered saline (pH 7.2). Ten volumes of the fixative described below were added to the sheep cell suspensions, and the mixture was left at room temperature overnight.

Alcohol-Formalin in Fixative Solution (pH 7.2)

40% formaldehyde	20	cc
95% ethyl alcohol	5	cc
Acid sodium phosphate, monohydrate	.4	g
Anhydrous disodium phosphate	.64	g
Distilled water	75	cc

Details of the fixation and washing procedure have been described(6). It was imperative that all traces of formaldehyde be removed. The cells were not tanned.

3. *Preparation of antigen.* Old tuberculin (Lederle),‡ 4 times standard strength, was diluted 1:10 in buffered saline solution (pH 7.2); 0.5 ml of tuberculin being diluted in 4.5 ml of buffered saline. To this, 1.0 ml of a 10% fixed cell suspension in buffered saline was added and incubated for 2 hours in a water bath at 37°C. The mixture was thoroughly shaken at approximately 15-minute intervals. This cell suspension was removed from the water bath and centrifuged. The cells were washed 3 times with 6 ml volumes of buffered saline and finally resuspended in 50 ml of buffered saline (0.2% cell suspension).

For comparison, fresh, unfixed erythrocytes were sensitized by the technic of Mid-

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dlebrook-Dubos(1,4); 0.5 ml of old tuberculin being diluted in 5.5 ml of buffered saline solution. To this, 0.1 ml of washed, packed fresh erythrocytes was added and incubated for 2 hours in a water bath at 37°C. The mixture was thoroughly shaken at approximately 15-minute intervals. This cell suspension was centrifuged at low speed (approximately 1,000 rpm) for 6 minutes. It is desirable to use a low speed in order to avoid a hard button of red cells. The cells were washed 3 times with 6 ml volumes of buffered saline and finally resuspended in 50 ml of buffered saline (0.2% cell suspension).

To compare the stability of these fixed, old tuberculin-coated cell suspensions under varying conditions, the final suspension of sensitized cells was made up to a 2% cell suspension in buffered saline and divided into several tubes. One-third of the tubes were stored in the refrigerator at 4°C, and one-third were quickly frozen at –70°C in a dry ice-ethyl alcohol mixture, then stored at –20° C. The remaining third were quickly frozen in the same manner, lyophilized, and stored at –20°C. When these fixed, sensitized cells were tested, they were resuspended as a 0.2% cell suspension in buffered saline.

4. *Absorption of patient's serum.* To 1.0 ml of buffered saline (pH 7.2) was added 1.0 ml of patient's serum, and the solution was inactivated at 65°C for 3 minutes. After cooling to room temperature, 0.2 ml of untreated washed packed sheep erythrocytes was added. The mixture was then incubated at 37°C in a water bath for 30 minutes. The cells were removed by centrifugation, and the serum stored at –20°C until assayed. The sera from 10 patients with active pulmonary tuberculosis were kindly provided by Dr. John McClement from the Chest Service, Bellevue Hospital, New York City.

The negative sera were obtained from persons with negative cutaneous reactions to tuberculin and with no evidence of chronic tuberculosis.

5. *Hemagglutination test.* Using special test tubes (13 × 75 mm)§ and racks, tests were set up with absorbed serum in 2-fold serial dilutions with buffered saline in a final volume of 0.4 ml. To each tube, 0.4 ml of the 0.2% suspension of the fixed or fresh sensitized sheep red cells was added. Controls included serum at the highest concentrations, to which 0.4 ml of a 0.2% suspension of *untreated,* washed sheep erythrocytes or *untreated* fixed erythrocytes was added. Other controls consisted of 0.4 ml of buffered saline to which 0.4 ml of 0.2% suspension of sensitized fixed or fresh cells were added.

The tubes were shaken and then incubated at 37°C in a water bath for 3 hours. Upon removal from the water bath, the tubes were reshaken and kept at room temperature overnight, to be read the following morning. The tests were read according to the usual criteria for the hemagglutination reaction. The tube containing sensitized cells in 0.4 ml buffered saline was used as a standard to judge the negative reaction.

Results. Eleven different batches of *old tuberculin*-sensitized cells were prepared at different times, but each contained the same concentration of fresh cells, fixatives, and antigen. For these, test sera from 10 different patients were used. The titers using the fixed cells tended to be slightly higher than those using fresh cells (Table I). The tubes containing sensitized fixed cells always showed distinct negative "buttons", whereas the tubes with sensitized fresh cells showed less sharply defined "buttons". The very satisfactory *reproducibility* of this technic for alcohol-formalin fixed, old tuberculin-sensitized erythrocytes is also shown in Table I.

The *stability* of 6 batches of fixed sensitized cells in the fluid state, in the frozen state, and in the lyophilized state over a period of 2 months is demonstrated in Table II. It is apparent that the titers remained essentially unchanged over this period when assayed with a single positive serum.

In the assays carried out with 5 sera from subjects who were negative by tuberculin skin test, distinct negative patterns were always observed. In addition to this, the tubes containing uncoated fixed cells in 1:2 dilutions always showed distinct negative patterns.

§ The tubes used for the hemagglutination test were specially designed by the Kontes Glass Co., Vineland, N. J.

TABLE I. Comparability of Results from 11 Batches of Fixed Sensitized Erythrocytes and 3 Batches of Fresh Cells.

Patient No.	Batches of fixed sensitized cells											Fresh cells		
	1	2	3	4	5	6	7	8	9	10	11	1	2	3
1	512*	256	256	256	256	512	256	256	256	256	256	128	128	256
2	0†	0	0	0	0	0	0	0	0	0	0	0	0	0
3	32	16	16	16	16	32	16	16	16	16	16	16	16	16
4	4	0	0	0	0	4	0	0	0	0	0	0	0	0
5	256	128	128	128	128	256	128	128	128	128	128	64	64	128
6	4	0	0	0	0	4	0	0	0	0	0	0	0	0
7	8	4	4	4	4	8	4	4	4	4	4	0	0	4
8	4	0	0	0	0	4	0	0	0	0	0	0	0	0
9	8	4	4	4	4	8	4	4	4	4	4	0	0	0
10	64	32	32	32	32	64	32	32	32	32	32	32	32	32

* The titers represent reciprocals of the serum dilution.
† A titer of less than 1:4 is indicated by 0.

Discussion. The Middlebrook-Dubos hemagglutination test is being by-passed by the internists because of other more advanced methods of diagnosis of pulmonary tuberculosis. However, because of the difficulties of diagnosis of ocular tuberculosis, particularly of tuberculous uveitis, the hemagglutination test might be of considerable value to the ophthalmologist.

The author agrees with Fine and Flocks (4) that in view of (a) the impossibility of demonstrating tubercle bacilli in most cases of ocular tuberculosis; (b) the low degree of parallelism between ocular and cutaneous sensitivity (as pointed out by Woods(5)); (c) the high percentage of positive skin tests in the general population; and (d) the danger of focal reaction from use of the tuberculin skin test, the hemagglutination test is a safer and perhaps more valuable diagnostic procedure than the skin test in diagnosis of tuberculosis. Whether this holds true for eye diseases, particularly uveitis, remains to be seen.

TABLE II. Stability of Frozen and Lyophilized Sensitized Fixed Cells.

Batch No.	Fluid		Frozen		Lyophilized	
	1 day	2 mo	1 day	2 mo	1 day	2 mo
1*	512†	256	512	512	256	256
2	256	128	128	128	128	128
4	256	N.T.‡	256	256	256	256
6	512	"	256	256	256	256
7	256	"	256	256	256	256
8	256	"	256	256	256	256

* Batch numbers correspond to those listed in Table I.
† The titers represent reciprocals of the serum dilution.
‡ N.T., not tested. A single positive serum was used throughout.

The use of fresh cells has the disadvantage of being time-consuming, and they are not always available. The use of antigen-coated, fixed erythrocytes in either fluid, frozen, or lyophilized state for detection of tuberculous antibodies, makes the hemagglutination test potentially available to even the smallest laboratories. Such a stable diagnostic reagent might, perhaps, be made and distributed by a central laboratory or commercial firm. Because of the need for stable reproducible diagnostic methods for investigation of uveitis and of other infectious diseases of the eye, it is felt that a wider use of such antigen coated fixed erythrocytes offers much promise.

Summary. 1. A method has been presented adapting ethyl alcohol-formalin fixed, sensitized erythrocytes to the Middlebrook-Dubos hemagglutination test for detecting antibodies against tuberculin. 2. The results using fixed cells with a number of sera were similar or more sensitive than those in which fresh cells were used. 3. Fixed tuberculin coated erythrocytes were successfully stored in the lyophilized state or in the frozen state for a period of 2 months without significant lowering of their sensitivity.

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Thromboplastic and Fibrinolytic Activities in Tissues of the Eye.* (27136)

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To elucidate further the physiological role of the hemostatic balance in the organism we have investigated the thromboplastic and fibrinolytic activities of tissues of the human and the pig eye.

Experimental. Materials. In 6 human eyes (from 4 patients) thromboplastic and fibrinolytic activities were investigated simultaneously. Because of the small size of the pig eye (obtained from freshly killed animals) 2 different series were required. The following components were separated and investigated: lens, vitreous body, cornea, sclera, choroid, retina. Only tissues which appeared to be normal were used.

Methods. Thromboplastic activity was estimated on freshly drawn citrated rabbit plasma and compared with a suspension of human brain as used for prothrombin estimations (after Owren). Samples were prepared by disintegration in a Potter homogenizer followed by freezing at −20°C. Activities of serial dilutions were estimated. Fibrinolytic activity was estimated (in triplicate) by the fibrin plate method (using 0.12% bovine fibrinogen clotted with bovine thrombin (Leo Pharmaceutical Prod., Copenhagen)) in serial dilutions obtained after extraction with KSCN and acid precipitation. Activity was expressed in units of a standard preparation of plasminogen activator prepared from pig heart. Details of methods in(1).

Results. Table I shows the thromboplastic activities recorded. The high activity of choroid and especially of retina is striking. No differences between human eyes and pig eyes are apparent. There is good agreement between estimations on 2 eyes of the same subject. None of the curves suggested presence of inhibitor material.

Table II shows the concentration of plasminogen activator in the tissues. Choroid ranges among the most active tissues so far investigated, whereas retina is in the low or medium range. The distribution of plasminogen activator in eye tissue follows a similar pattern in man and pig.

Though the human material is limited, the uniformity of the data warrants some tentative suggestions. The very low thromboplastic activities and fairly low fibrinolytic activities of the sclera are similar to the conditions previously observed in human joint tissues (fibrous capsular tissue, synovial membrane(2). From a pathological viewpoint the findings could explain the occurrence of endovitreous hemorrhagic processes followed by slow reabsorption. The extremely high fibrinolytic activity of the choroid is not surprising in view of previous findings of high fibrinolytic activity in highly vascularized connective tissue(3), including the meninges(4). Coupled with a high thromboplastic activity this suggests that the choroid is

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