Stability of Fluorescent Antibody Conjugates Stored Under Various Conditions

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Two experiments were carried out to determine the stability of fluorescent antibody conjugates. In experiment 1, *Francisella tularemia* conjugates in the lyophilized state retained their original staining titer for 1,294 days when stored at 25, 4 to 5, and -20°C ; at 37°C the conjugates were stable for at least 65 days. In the liquid state at pH 7.4 and 8.0 these conjugates were stable for 1,294 days at 4 to 5 and -20°C , whereas those stored at 25°C remained stable through days 473 and 160 of storage, respectively, after which the staining titer gradually dropped. In experiment 2 five previously lyophilized conjugates were rehydrated with three different diluents and stored at 4 to 5°C for up to 600 days at their working dilutions. All of these conjugates retained their original staining titer during the test period except an anti-human globulin conjugate rehydrated with phosphate-buffered saline. Recommendations are made for the long-term storage of fluorescent antibody conjugates.

The stability of in vitro diagnostic products has assumed additional significance since the Food and Drug Administration established statutory requirements (1) that expiration dates, or some other indication of stability, must be placed on the label of each product sold by commercial manufacturer. In addition, this law also requires that the label on a product that must be manipulated before use, for example, diluted or rehydrated from a dried state, must also state the length of time the user can expect the final product to remain stable. Thus, definitive information on the stability of these products stored under various conditions is important to both commercial manufacturers and to the consumer. Furthermore, many laboratories prepare some reagents for their own use, and this type of information would be beneficial to them.

There have been several reports on the stability of fluorescent antibody (FA) conjugates, but the studies have been rather limited in scope and the time covered. Winter and Moody (5) reported that an FA conjugate for *Yersinia pestis* was stable for at least 1 year when stored lyophilized at room temperature or when frozen in a deep freeze or in the liquid state at 0 to 5°C . Lewis et al. (3) found that an FA conjugate of anti-human globulin was stable for at least 50 days when stored at either 4 or -20°C . Roberts et al. (4) reported that an anti-human conjugate was stable when stored frozen at -60°C or lyophilized and stored at -20°C for 6.5 and 4

months, respectively. The stability of the same conjugate stored at 4°C was dependent upon the type of buffer used, its molarity, and its pH.

Two studies on the stability of FA conjugates are described in this report. (i) *Francisella tularensis* conjugates were stored either lyophilized, frozen, or in the liquid state at two different pH's and four different temperatures for up to 1,294 days, and (ii) five different FA conjugates previously lyophilized for various periods of time were reconstituted with three different diluents and stored at 4 to 5°C for up to 600 days.

These conjugates were prepared for use in the diagnostic laboratories at the Center for Disease Control (CDC) and are supplied, in limited quantities, to State Health Department Laboratories and to diagnostic laboratories and other Federal agencies. Before distribution they were evaluated by two laboratories at CDC to insure that they met the CDC minimum requirements for potency and specificity. It is felt that these conjugates are comparable in physicochemical properties to those distributed by most commercial manufacturers of diagnostic reagents.

MATERIALS AND METHODS

Conjugates used. An *F. tularensis* conjugate was used for experiment 1. The preparation of this conjugate has been previously described (2). It contained 10 mg of protein per ml and had a fluorescein to protein ratio of 8 (micrograms of fluores-

cein per milligram of protein). The bulk conjugate was divided into two aliquots, one adjusted to pH 7.4 and the second to pH 8.0. The conjugate was then dispensed in 2-ml amounts into Wheaton vials, and half of the vials at each pH were lyophilized and sealed under vacuum with vinyl stoppers. The conjugate in the liquid state was also sealed with vinyl stoppers. The vials containing the liquid and lyophilized conjugates were further subdivided and stored at one of the following temperatures: 37, 25, 4 to 5, and -20 C. For stability testing two vials stored under each of the above conditions were removed at various times, and the homologous staining titer was determined.

For experiment 2, the following five conjugates were used: *F. tularensis*, *Y. pestis*, *Escherichia coli*, group A streptococci, and anti-human globulin used in the indirect FA (IFA) test for toxoplasmosis. These conjugates had been prepared at different times in our laboratory and stored lyophilized under vacuum at 4 C (Table 1). Each conjugate was prepared by procedures routinely used at CDC at the time of their preparation. Briefly this consisted of three precipitations of hyperimmune antiserum with 50% ammonium sulfate and suspension of the globulin fraction in distilled water followed by dialysis against 0.01 M phosphate-buffered saline, pH 7.6 (PBS), to remove the residual ammonium sulfate. The globulin was adjusted to pH 9.5 and reacted with fluorescein isothiocyanate at room temperature for 2 h. The unreacted dye was then removed by dialysis against PBS. Enough vials of each conjugate were rehydrated with one of three diluents so that when the rehydrated conjugates were pooled there was enough for testing for at least 1 year. The following diluents were used: (i) that recommended on the vial, either PBS or distilled water; (ii) 0.3 M glycine, pH 7.2; and (iii) 1% aqueous solution of bovine serum albumin. Each diluent for the anti-human globulin contained 0.2% Evans blue as a counterstain for the *Toxoplasma gondii* organism used as antigen. The conjugates had been dispensed so that when they were rehydrated to the volume given on the vial, they were diluted to the recommended working dilution (Table

1.) The recommended working dilution of each conjugate, except the anti-human globulin, was at least one twofold dilution below the highest dilution giving a 3 to 4+ staining. The working dilution of the anti-human globulin conjugate was the highest dilution, giving a 1+ staining in the IFA test for toxoplasmosis using a positive human serum with an IFA titer of 1:1,024. A lyophilized vial of each conjugate was rehydrated with the diluent recommended on the vial (Table 1) each time a conjugate was tested. This served as a control to insure that the antigen used at that time gave a satisfactory staining reaction. Serial twofold dilutions of each conjugate, except the anti-human globulin, were prepared with PBS, and the highest dilution giving a 3 to 4+ staining was used as the end point. The anti-human globulin conjugate was used at its working dilution, the end point being the highest dilution of a positive human serum that gave a 1+ staining reaction in the IFA test. A difference of one twofold dilution in the staining titer at different testing periods was not considered significant. Pertinent information on the lyophilization and rehydration of these conjugates is given in Table 1.

For experiment 1 new slides were prepared each time the conjugate was tested. For experiment 2 one strain of each homologous organism was used as a test antigen. Slides containing smears of *F. tularensis*, *Y. pestis*, and *T. gondii* were prepared at the beginning of the experiment, stored at -20 C, and used for all the tests. Slides for *E. coli* and group A streptococci were prepared each time the conjugates were tested. All slides were examined on a Leitz microscope equipped with an HBO 200 mercury vapor lamp, a Schott BG-12 primary filter, and a Schott OG-1 barrier filter.

RESULTS

Experiment 1. The results of this study are given in Table 2. The conjugates stored at 37 C were stable for at least 63 days, except those stored in the liquid state at pH 8.0. There was a significant drop in the titer of the latter conjugate between days 35 and 63 of storage

TABLE 1. *Lyophilization and rehydration conditions for FA conjugates used in experiment 2*

Conjugate	Animal	Lyophilization conditions					Rehydration conditions		
		mg of protein per ml	F/P ^a ratio	Vol dispensed per vial (ml)	Original diluent	Date lyophilized	Vol added (ml)	Recommended diluent	Final dilution
<i>Yersinia pestis</i>	Rabbit	10	10	0.5	PBS	2/25/70	1.0	PBS	1:2
<i>Francisella tularensis</i>	Horse	12.5	7.5	0.1	PBS	2/2/66	1.0	PBS	1:10
<i>Escherichia coli</i>	Rabbit	10	8.0	0.5	PBS	11/21/62	10.0	Water	1:20
Group A streptococci	Rabbit	5	13.7	1.0	NRS ^b	9/20/72	2.0	Water	1:50
Anti-human globulin	Goat	11.5	15.0	0.2	NGS ^c	8/28/72	2.0	PBS ^d	1:300

^a F/P, Fluorescein to protein.

^b Conjugate diluted 1:30 with normal goat serum before lyophilization.

^c Diluent contained 0.2% Evans blue.

and in all of the conjugates by day 119 of storage. Based on these results the conjugates stored at the other temperatures were not tested until day 168 after the initial testing at day 0. At 25 C there was no loss in the titer of the lyophilized conjugate during the 1,294 days of storage. The conjugates stored at 25 C in the liquid state at pH 7.4 retained their titers through day 473 of storage, and then, during the rest of the test, the titer dropped fourfold. The liquid conjugates stored at 25 C and pH 8.0 retained their titers for at least 160 days; the titers then gradually dropped, reaching an un-

acceptable level by day 1,294 of testing. All of the conjugates stored at 4 to 5 C and -20 C retained their original titer during the 1,294 days of testing.

Experiment 2. To determine the stability of lyophilized FA conjugates after rehydration, five conjugates that had been lyophilized and stored at 4 C for different periods of time (Table 1) were rehydrated with three different diluents, stored at 4 to 5 C, and checked with the homologous antigen every 2 months for up to 600 days. The results (Table 3) show that all the conjugates except the anti-human globulin

TABLE 2. Staining titers of liquid and lyophilized *F. tularensis* conjugates stored at different pH and temperatures

Storage conditions ^a	Storage temp (C)	Conjugate staining titers ^b								
		0 ^c	35	63	119	168	242	473	640	1294
Lyo pH 7.4	37	160	80	80	20	20	NT	NT	NT	NT
Lyo pH 8.0		160	80	80	20	20	NT	NT	NT	NT
Liq pH 7.4		160	80	80	20	20	NT	NT	NT	NT
Liq pH 8.0		160	80	20	10	<10	NT	NT	NT	NT
Lyo pH 7.4	25	160	NT	NT	NT	160	80	160	80	80
Lyo pH 8.0		160	NT	NT	NT	160	160	160	80	80
Liq pH 7.4		160	NT	NT	NT	160	80	80	40	40
Liq pH 8.0		160	NT	NT	NT	160	40	40	20	<10
Lyo pH 7.4	4 to 5	160	NT	NT	NT	160	160	80	160	160
Lyo pH 8.0		160	NT	NT	NT	160	160	160	160	160
Liq pH 7.4		160	NT	NT	NT	160	160	80	80	160
Liq pH 8.0		160	NT	NT	NT	160	160	160	80	80
Lyo pH 7.4	-20	160	NT	NT	NT	80	80	160	160	160
Lyo pH 8.0		160	NT	NT	NT	160	160	160	80	160
Liq pH 7.4		160	NT	NT	NT	80	80	160	160	160
Liq pH 8.0		160	NT	NT	NT	160	80	160	160	160

^a Lyo, Lyophilized; Liq, liquid.

^b Titers, End-point dilution factor of highest dilutions giving 3 to 4+ staining. NT, Not tested.

^c Days of storage.

TABLE 3. Staining titers of previously lyophilized FA conjugates rehydrated in different diluents and stored at 4 to 5 C

Rehydration diluent ^a	Conjugate staining titers ^b									
	Group A streptococci		<i>Escherichia coli</i>		<i>Yersinia pestis</i>		<i>Francisella tularensis</i>		Anti-human globulin ^c	
	0 ^d	600	0	560	0	591	0	591	0	591
0.3 M glycine	8	8	8	4	512	256	8	8	1,024	1,024
1% BSA	8	8	8	4	512	256	8	8	2,048	2,048
Diluent	8	4	2	4	256	256	4	8	512	64
Control	8	8	2	2	256	256	4	8	512	1,024

^a BSA, Bovine serum albumin. Diluent recommended on vial (see Table 1). Controls, A lyophilized vial of the conjugate rehydrated at each testing interval.

^b Titer of antihuman serum is end-point dilution factor of highest dilution of human serum from a case of toxoplasmosis giving a 1+ staining titer of other conjugates are end-point dilution factors of highest dilution giving 3 to 4+ staining.

^c All diluents contained 0.2% Evans blue.

^d Days of storage.

retained their original titer in all three diluents during the entire test period. The anti-human globulin conjugate rehydrated with PBS containing 0.2% Evans blue originally stained *T. gondii* cells that had been reacted with a positive control toxoplasmosis human serum diluted 1:512 to 1:1,024. After the rehydrated conjugate had been stored for 591 days, the staining titer for the same positive control serum dropped to 64, whereas the control conjugate still had a titer of 512 to 1,024. The staining activity of the conjugate dropped somewhat after storage for 40 days, and after storage for 96 days, the conjugate was not satisfactory for use in the IFA test. This same conjugate that had been rehydrated with either 0.3 M glycine or 1% bovine serum albumin retained its original titer after storage for 591 days. Upon rehydration the latter conjugate was diluted 1:300, which was the recommended working dilution.

DISCUSSION

The data obtained in experiment 1 demonstrates the stability of FA conjugates stored at 4 to 5 C and -20 C in either the liquid or lyophilized state. This data is further supported by the results obtained in experiment 2 with *E. coli* and *F. tularensis* conjugates that had been lyophilized and stored at 4 C for approximately 10 and 6 years, respectively, without any observable loss in the original staining titer (Table 1). At 25 C the lyophilized conjugate also remained stable during the 1,294-day test period, whereas the conjugates in the liquid state gradually dropped in titer particularly when stored at pH 8.0 rather than pH 7.4. At 37 C the titer of the conjugates in the liquid state at pH 8.0 also dropped more rapidly than those stored at pH 7.4.

The stability of conjugates that were rehydrated from the lyophilized state and stored at 4 C for up to 600 days (experiment 2) has a very practicable implication, particularly to those laboratories that perform a limited number of FA tests and have to store conjugates in the liquid state for long periods. Even though these conjugates were stored at the working dilution which reduced the protein content to a rather low level, all but one retained their original titers during the test period. For example the *E. coli* and *F. tularensis* conjugates were stored at approximately 0.5 and 1.25 mg of protein per ml, respectively, without any significant loss in titer. The only conjugate used in experiment 2 that had a drop in titer during the time of the study was the anti-human globulin rehydrated with PBS for use in the IFA test for toxoplasmosis. Before lyophilization this latter conjugate was diluted 1:30 with nor-

mal goat serum and when rehydrated was further diluted 1:10 for a final dilution of 1:300. This, in effect, reduced the original globulin solution from 11.5 mg of protein per ml to approximately 0.04 mg of protein per ml. Although these studies were not designed to determine the effect of the protein concentration on the stability of FA conjugates, the results suggest that at very low levels of antibody protein, the presence of large amounts of normal serum does not protect the antibody activity with PBS as the diluent.

Another observation from experiment 2 was that conjugates rehydrated with either 0.3 M glycine or 1% bovine serum albumin had very little, if any, background staining, whereas those rehydrated with either water or PBS frequently had a definite greenish hue in the background, suggesting that the former two diluents not only stabilized the conjugates but also quenched the nonspecific background staining. This is particularly beneficial in tests, such as the indirect FA test for toxoplasmosis, where the titer of a positive unlabeled serum is a 1+ staining reaction with the anti-human globulin conjugate.

From these studies the following three recommendations can be made for storage of FA conjugates. (i) Lyophilized conjugates may be stored for at least 1,294 days at 25 C or lower without a significant loss of titer. However, the rather rapid drop in titer of conjugates stored at 37 C suggests that storage at 4 to 5 C or lower is preferable. (ii) Conjugates in the liquid state should be stored at 4 to 5 C or -20 C. (iii) Lyophilized conjugates that are diluted to the working dilution when rehydrated may be stored for at least 600 days at 4 to 5 C. If a conjugate is used at a high working dilution, 0.3 M glycine or 1% bovine serum albumin should be used as a diluent rather than PBS.

LITERATURE CITED

1. Federal Register. 1973. In vitro diagnostic products for human use. Labeling requirements and procedures for development. Fed. Regist. 38:7096-7102.
2. Green, J. H., R. C. Bolin, R. K. Carver, H. Gross, N. Pigott, and W. K. Harrell. 1970. Preparation of agglutinating antisera and fluorescent-antibody conjugates against *Pasteurella tularensis* in equines. Appl. Microbiol. 19:894-897.
3. Lewis, V. J., W. L. Jones, J. B. Brooks, and W. B. Cherry. 1964. Technical considerations in the preparation of fluorescent antibody conjugates. Appl. Microbiol. 12:343-348.
4. Roberts, M. E., J. N. Miller, T. C. Pringle, and G. F. Binnings. 1968. Shelf life of fluorescent treponemal antibody-absorption test reagents. J. Bacteriol. 96:1507-1511.
5. Winter, C. C., and M. D. Moody. 1959. Rapid identification of *Pasteurella pestis* with fluorescent antibody. II. Specific identification of *Pasteurella pestis* in dried smears. J. Infect. Dis. 104:281-287.