

Stability and behavior of reaginic antibodies: Effects of freezing, thawing, and lyophilizing on skin-sensitizing activity of reaginic sera

Frank Perlman, M.D., and Laurence L. Layton, Ph.D.**
Portland, Ore., and Albany, Calif.*

This is one in a series of studies on the behavior and stability of reaginic antibodies subjected to various physical, chemical, and thermal influences. Observations were made on the effect of freezing, thawing, and storing, in the lyophilized state, sera of various ages from a few hours to more than four years. These observations provided a basis for acceptance of the reliability of our entire serum banks used in the successive studies. Both human volunteers and lower primates were employed as recipients for the passive transfer studies. The sera used contain reaginic antibodies for a variety of allergens. Those containing reagin for penicillin were most valuable for quantitation, since challenge at a remote site permitted the presumption of delivery of equal quantities of allergens to all the sensitized sites, thus eliminating the element of trauma and error of measurement, faults inherent in the direct challenge. Results indicate that lyophilization as well as storage of serum at subfreezing temperature preserved the reaginic antibodies equally well for a number of years (perhaps better with lyophilized serum). Repeated freezing and thawing, and even allowing the serum to stand in liquid state for hours to several days, did not significantly alter the reaginic titer, as measured by passive transfer test on the monkey. These results will make more acceptable our succeeding studies and permit our expanding our serum banks for long-range immunological studies with reaginic antibodies.

Since reaginic antibodies play a dominant role in the study and demonstration of human allergy, there has been increased interest in identifying them

Received for publication Aug. 18, 1966.

*Division of Immunology, Allergy, and Infectious Diseases, University of Oregon Medical School, Portland.

**Western Regional Research Laboratory (a laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture), Albany, Calif. 94710.

among the immune globulins and isolating them in quantities adequate for study of their physical, chemical, immunological, and biological properties. The lability of reagin requires that greater care be exercised in handling such sera than is often employed in the study of other antibodies. The thermolability of reaginic activity is readily demonstrated,^{1, 3} and storage under ordinary refrigeration temperatures of 0 to 4° C. will result in its dissipation in weeks to months.⁴ The ability of reagin to withstand other physical, chemical, and thermal influences has not as yet been clearly documented.

As our large serum banks become even larger and older, we find it important to know how stable our stored reaginic sera are and how reliable and reproducible our various experiments will be. Therefore, a series of studies is in progress in our respective laboratories to determine the stability of reaginic antibodies in the whole serum exposed to various influences. These investigations began with the study³ of a number of sera stored at -20° C. and tested on both humans and monkeys. It is being continued with the use of aliquots of the same sera subjected to various physical, chemical, and thermal changes.

This report concerns itself primarily with the effect on reagin titer of lyophilization as well as with repeated freezing, thawing, and storing above freezing temperatures. Although a large number of reaginic sera were screened, only several are here reported to illustrate various observations. Two such sera containing reagin for penicillin are reported in detail, since the sensitized sites could be challenged by injection of the allergen at a remote point.⁵ This permitted the consideration that equal quantities of penicillin reached all sensitized areas. These "indirect" Prausnitz-Küstner type reactions also provided the advantage of reducing the factors of trauma and other nonspecific irritation, which are inevitable with direct challenge to the sensitized sites. Furthermore, such "indirect" challenge on humans paralleled the method found most useful in passive transfer testing on the monkeys.^{3, 6}

MATERIALS AND METHODS

Serum

The reaginic sera were obtained from allergic patients who gave strong skin test reactions and who were clinically sensitive to the challenging allergens employed. Each serum was obtained in quantities of 10 to 20 ml., was divided under sterile conditions into aliquots of 1 to 2 ml., and frozen within 4 hours after the blood had been drawn. All samples were then stored at a temperature of -20° C. until thawed for use. Those samples which were lyophilized were placed in dry ice for an hour, then put uncapped in a vacuum jar, after which lyophilization was carried out for 24 hours in a vacuum adequate to maintain a solid state. The vials were then capped under sterile conditions without any attempt at maintaining vacuum and were stored at ordinary refrigeration temperature of 4° C. None of the sera was preserved chemically or passed through a sterilizing filter. After thawing or reconstituting with distilled water, the sera were used on human volunteers within 48 hours. The allergic serum donors had been questioned carefully, and none gave a history of hepatitis or recent obscure illnesses. The serologic tests for syphilis were negative.

Allergens

These allergens included house dust, dog hair, grass pollens, and cockroach extract. Allergens were currently in use in our clinic and had been employed in testing the allergic serum donors. The concentration of these allergens was calculated on the basis of weight volume, except for penicillin which was recorded in units.

The penicillin G potassium (Pen GK) was freshly prepared for each experiment and was the only form of penicillin employed in the challenges, since the sera had previously been challenged with penicilloyl-polylysine and with 6 amino-penicillanic acid, without significant reactions. One of the penicillin-allergic patients (M. R.) failed to react to these various derivatives of penicillin, even on direct testing, but yet gave a systemic reaction on scratch test with Pen GK.

Subjects for passive transfer

Recipients of the sera included both human volunteers and subhuman primates (*Macacus irus* and *Macacus speciosus*). The human volunteers ranged in age from 23 to 40 years, were in vigorous health, and gave no history of allergic disorders. They were screened by direct intradermal skin testing with the allergens employed, and none had positive wheal-or-flare reactions.

The subhuman primates included the *Macacus* monkeys. All were young specimens of approximately one year of age, weighing 2½ to 4 pounds.

Passive transfer procedures

On the human volunteers, the back provided a uniform skin surface for sensitizing sites, each of which was prepared by injecting intradermally 0.10 ml. of serum in various dilutions, as indicated in each experiment. The sites were marked as to location and as to direction of insertion of the needle. The first challenge with the allergen was made after 48 hours, with the introduction again intradermally of 0.02 ml. as nearly as possible in the direction and at the site of the previously placed reaginic serum. The challenging doses of dust, dog hair, grass pollen extract, and cockroach extract were in concentrations tenfold greater than that which produced wheal-and-flare reactions in the allergic serum donor. However, with penicillin a remote site was used for "indirect challenge," and in each instance a small quantity of the allergen (200 units or approximately 0.12 meg.) provided optimum allergen excess. This quantity was arbitrarily chosen but was based on a number of previous successful experiments with these sera.

On the monkeys, sites were sensitized on the face and ventral surface of the trunk and thighs. After 48 hours, 2.5 ml. of 0.5 per cent Evans blue dye was injected intravenously, and, after 15 minutes to 1 hour, challenges with the allergens were performed. In the case of dog hair this was accomplished by injection of the allergen into the saphenous veins. With Pen GK, the placement of a small, unmeasured quantity of powered material into the animal's mouth produced reaction at the sensitized skin sites within 2 to 5 minutes.

Aliquots of the same sera of J. H. (Experiment I) and of M. R. (Experiment II) used on the human volunteers also were tested on the monkeys. Results paralleled each other both qualitatively and quantitatively. These two sera were fur-

ther subjected to a variety of physical and thermal influences for passive transfer to the monkey, and details of these results are described in Fig. 2.

OBSERVATIONS

The observations and results are presented in detail in the summaries of experiments on two of the human volunteers each sensitized with a different serum and one of the monkeys who was sensitized with both sera. Brief summaries on other sera are also offered.

Experiment I

Objectives: (1) evaluation of the stability of three different reaginic antibodies in a single serum in frozen and in lyophilized states; (2) successive exhaustion of reagins for each individual allergen.

Serum of J. H.: drawn, frozen, and lyophilized on March 25, 1965.

Allergens: penicillin G potassium, house dust, and dog hair.

Conclusions: (1) Three different reaginic antibodies in a single serum differing in titer were not detectably affected by lyophilization (Table I). (2) Exhaustion of the site for one allergen did not affect the reaction on challenge with a second allergen, and exhaustion to the second did not affect reaction on challenge with a third allergen.

Table I. *Observation in Experiment I*

Allergen sites sensitized (7-15-65)	Penicillin GK				House dust				Dog hair	
	First challenge (7-17-65)		Second challenge (7-20-65)		First rechallenge (7-20-65)		Second rechallenge (7-25-65)		Further challenge (7-27-65)	
	Frozen	Lyophilized	Frozen	Lyophilized	Frozen	Lyophilized	Frozen	Lyophilized	Frozen	Lyophilized
<i>Dilution</i>										
Full strength	4+	4+	—	—	4+	4+	3+	3+	4+	4+
1:3	3+	3+	—	—	4+	4+	2+	2+	3+	3+
1:9	2+	2+	—	—	3+	3+	2+	2+	3+	3+
1:25	±	±	—	—	3+	3+	2+	2+	3+	3+
1:125	—	—	—	—	2+	2+	+	±	3+	2+
1:500	—	—	—	—	2+	2+	±	±	±	+
1:1000	—	—	—	—	±	+	±	±	±	±
Control	—*		—*		—†		±†		±†	

Sites were prepared with serial sera dilutions from full strength to 1:1000 and after 48 hours were challenged indirectly* with Pen GK, 200 units, lighting up sites in dilutions through 1:9. Second challenge in 72 hours indicated exhaustion of reagin for penicillin at all sites. Direct† challenge with house dust gave a strong reaction in dilutions through 1:500. Rechallenge in 48 hours indicated exhaustion of reagin for house dust. However, 48 hours later, or 12 days after sensitizing the sites, further challenge with dog hair gave reactions in dilutions of 1:125.

*Pen GK, 20,000 units, was injected only at control sites, thus the sensitized sites reacted by "indirect challenge."

†House dust and dog extracts were injected directly into each sensitized site as well as into the control sites.

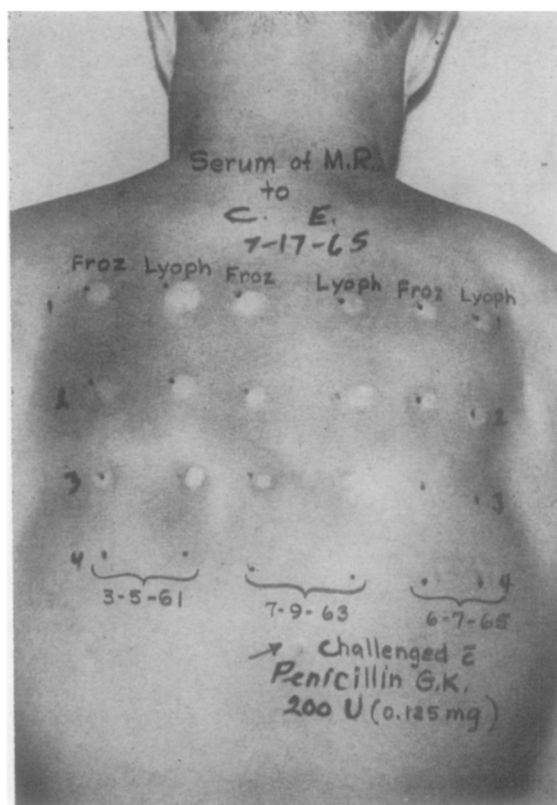


Fig. 1

Sera of various ages (1961, 1963, 1965) of a single penicillin-sensitive patient (*M. R.*) in both frozen and lyophilized states were used (Experiment II). Dilutions of sera varied from (1) full strength, (2) 1:5, (3) 1:20, and (4) 1:100. Challenge was made with Pen GK, 200 U., at a single remote site. This same serum was used on the cheeks of the Macaque monkey in Experiment III.

Experiment II

Objective: titration of reaginic antibodies in three serum samples from a single allergic donor drawn at intervals of 4 years, 2 years, and 1 month.

Sera of *M. R.*: (A) drawn and frozen March 5, 1961; lyophilized March 15, 1961; (B) drawn and frozen July 9, 1963; lyophilized July 24, 1963; (C) drawn and frozen June 7, 1965; lyophilized July 13, 1965.

Conclusions: (1) The serum of 1965 had a lower reaginic titer than those of 1963 and 1961, probably due to a drop in circulating antibodies as the result of the avoidance of penicillin (Fig. 1). (2) Lyophilization did not detectably alter the titers. (3) Serum stored in frozen state for more than 4 years had a slightly lower titer than that of an aliquot of the same serum that had been lyophilized and stored also for more than 4 years.

Experiment III

Objectives: (1) use of Macaque monkeys for study of the stability of the same reagin used in the human passive transfer experiments; (2) effects on the two sera of J. M. and M. R. subjected to further freezing, thawing, and storing at room temperature.

Procedure and observations (Fig. 2).

Series X: Serum frozen March 5, 1961, was thawed and used Jan. 10, 1966. This gave moderate reaction in the 1:2 dilution and gradually lost its reactivity at 1:16.

Series Y: Serum frozen March 5, 1961, lyophilized March 15, 1961, and re-

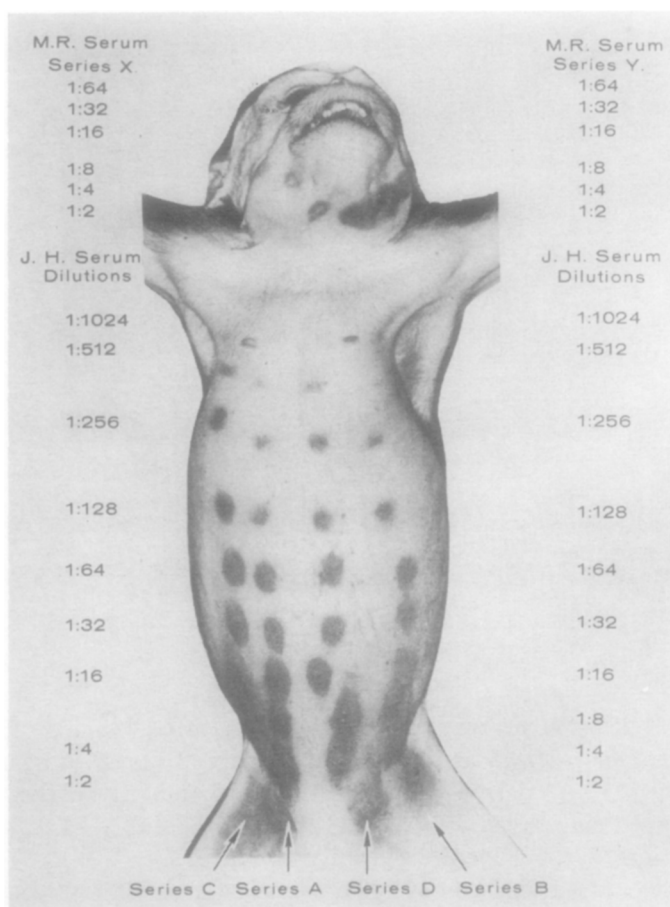


Fig. 2

Sera of varying dilutions which were used in previous human experiments, I and II. The sera of *M. R.* on the face of the monkey (*series X and Y*) were frozen or lyophilized once and reconstituted. Those of *J. H.* on the monkey's trunk (*Series A, B, C, D*) were subjected to additional physical influences as described in the experiment. The monkey shown is *Macacus speciosus* (stumptail).

constituted July 15, 1965, for human Experiment II, was frozen and thawed and used on Jan. 10, 1966. In spite of the freeze-dry processing, this serum gave strong positive reaction in a 1:2 dilution and gave a moderate reaction even in a dilution of 1:64.

Series A: This serum sample was frozen March 25, 1965, thawed, and then refrozen and thawed 22 times (from -60° C. to $+25^{\circ}$ C. in 15 minute cycles). It gave strong positive reactions in dilutions of 1:256 and moderate reaction in dilutions of 1:512.

Series B: This serum sample lyophilized March 25, 1965, and reconstituted Jan. 10, 1966, gave strong positive reaction in dilution of 1:512.

Series C: This serum sample lyophilized March 25, 1965, reconstituted Jan. 5, 1966, stored at 30° C. for 4 hours and at 8° C. for 72 hours, then frozen, and finally thawed for use on Jan. 10, 1966, gave strong positive reaction in dilution of 1:512.

Series D: This serum sample frozen March 28, 1965, and stored until thawed for use Jan. 10, 1966, gave positive reaction in dilution of 1:512. Samples of this serum were heated at 56° C. for 1 hour after which it failed to sensitize monkey skin.

Experiment IV (Macaque monkeys)

Objective: comparison of reagin titer of fresh serum before and after one freeze-thaw cycle.

Serum of M. K.*: untreated patient severely allergic to cat and house dust; serum drawn Feb. 8, 1965; one aliquot chilled, others frozen, and several lyophilized. Total elapsed time from bleeding to sensitization of *Macacus irus* monkey³ with fresh and frozen sera was less than 6 hours. The lyophilized sample was compared with the frozen serum 18 months after the blood was drawn.

Observations: Comparable reactions occurred in the fresh, the frozen, and the lyophilized samples with 4+ in a dilution of 1:2 reduced to 1+ in a dilution of 1:64 and negative beyond this (Table II).

Conclusions: (1) Reaginic titer is not appreciably affected by a single freeze-thaw cycle. (2) Eighteen months after first comparison the titer of lyophilized

*Patient M. K. was skin tested by Thomas W. Green, M.D., Ph.D., who also drew the blood sample on Feb. 8, 1965.

Table II. Observations on Experiment IV. Comparison of fresh serum, fresh-frozen serum, and lyophilized serum reagin titer of serum from patient M. K.

Treatment	Dilution						
	1:2	1:4	1:8	1:16	1:32	1:64	1:128
Fresh serum	4+	4+	4+	3+	2+	1+	0
Fresh-frozen serum	4+	4+	4+	4+	2+	1+	0
Lyophilized serum*	4+	4+	4+	4+	3+	1+	0

The lyophilized sample was compared with frozen serum 18 months after the blood was drawn.

serum and frozen serum showed comparable titers and were comparable to the original fresh serum.

Additional observations: This serum was heated at 56° C. for one hour after which it failed to sensitize the monkey skin.

Additional experiments

In testing a number of other sera subjected to freezing, thawing, and lyophilizing, our observations duplicated those described in Experiments I, II, III, and IV. Several of these observations are summarized in Tables III and IV.

Table III. *Observations on two sera in both frozen and lyophilized states*

Sites sensitized (7-15-75)	House dust					
	First challenge (7-17-65)		Second challenge (7-20-65)		Third challenge (8-22-65)	
	Frozen	Lyophilized	Frozen	Lyophilized	Frozen	Lyophilized
<i>Serum of D. C.: Dilution</i>						
1:5	4+	4+	2+	2+	—	—
1:25	3+	3+	—	—	—	—
1:125	2+	2+	—	—	—	—
1:500	1+	1+	—	—	—	—
1:1000	—	—	—	—	—	—
<i>Control</i>	±	—	—	—	—	—
<i>Serum of R. F.: dilution</i>						
1:5	4+	4+	—	—	—	—
1:25	3+	3+	—	—	—	—
1:125	3+	3+	—	—	—	—
1:500	2+	2+	—	—	—	—
1:1000	1+	1+	—	—	—	—
<i>Control</i>	—	—	—	—	—	—

Serum of D. C., frozen Feb. 10, 1965, and lyophilized July 7, 1965, and serum of R. F., frozen May 21, 1965, and lyophilized July 7, 1965, had comparable titers of reagin for house dust. In each case the frozen and lyophilized samples reacted in approximately the same titers and were both readily exhausted.

Table IV. *Observations on three sera, containing reagin in low titers, showed no difference between the frozen and lyophilized states*

Sites sensitized*	D. A. (Cockroach allergen)		Wm. G. (Grass pollen)		A. H. (Grass pollen)	
	Frozen	Lyophilized	Frozen	Lyophilized	Frozen	Lyophilized
<i>Dilution</i>						
1:5	3+	3+	3+	3+	3+	3+
1:25	2+	3+	3+	3+	1+	1+
1:125	2+	2+	1+	1+	—	—
1:500	1+	—	±	—	—	—
1:1000	—	—	—	—	—	—
<i>Control</i>	—	—	—	—	±	—

Serum of Wm. G. had been lyophilized Aug. 6, 1963, (three years before use) while that of A. H. had been lyophilized July 7, 1965, (10 days before use). Both serum donors were clinically sensitive to grass pollen.

*Sites were sensitized July 17, 1965, and challenged on July 20, 1965.

COMMENTS

Stability of reaginic antibodies was judged on the basis of unchanged titers measured by passive transfer to humans and monkeys. This is at best an inaccurate and somewhat crude system for measurement since not only do recipients vary in reactions but also, on a single recipient, different areas will vary in degree of reactivity. Efforts were made in these experiments to choose comparable sites on both man and monkey for the same serum which had been subjected to varying physical changes. The consistency of our findings would indicate that reagin is much more stable than it is generally purported to be.

It is quite possible that the use of whole serum provided some protection against these physical influences. As the gamma globulins containing these reaginic antibodies are purified, there is some evidence of reduced stability, especially in storage at ordinary refrigeration temperature. As higher degree of purification is developed, measurement of their stability will require re-evaluation. As evidence increases that reagin represents a heterogeneous group of antibodies when measured by physicochemical methods, the stability of one may differ from that of another. Our current studies with certain chemicals added to sera with the use of passive transfer methods strongly suggest that reaginic antibodies are indeed a heterogeneous group. For example, skin-sensitizing antibodies to penicillin appear to be destroyed by these chemical agents; yet in the same sera those antibodies to epidermals and dusts are only slightly affected.

As an aside, two interesting observations deserve to be pointed out. Passive transfer to humans and to monkeys paralleled each other both qualitatively and quantitatively in these experiments. It is also interesting to note that exhaustion for one reagin did not exhaust the capacity of the tissue site to react to challenge with another allergen. This observation does not concur with those made by Pruzansky and co-workers⁷ but was repeatedly demonstrated with the serum of J. H. on one of the authors (F. P.) and on several other human recipients.⁵

REFERENCES

1. Richter, M., Harter, H. F., Sehon, A. H., and Rose, B.: Studies on Ragweed Pollen. III. Estimation of the Minimum Number of Allergens in Water Soluble Extract of Ragweed Pollen and a Critical Evaluation of the Neutralization Technique. The Demonstration of Two Different Reagins in Sera of Ragweed Sensitive Individuals, *J. Immunol.* **79**: 13, 1956.
2. Sehon, A. H., Harter, J. G., and Rose, B.: The Localization of Skin-Sensitizing Antibody in the Sera of Ragweed-Sensitized Individuals by Electrophoresis, *J. Exper. Med.* **103**: 679, 1956.
3. Layton, L. L., Yamanaka, E., Greene, F. C., and Perlman, F.: Atopic Reagins to Penicillin, Pollens, and Seeds: Thermolability, Titer and Persistence in the Skin of Passively Sensitized Macaque Monkeys, *Internat. Arch. Allergy* **23**: 87, 1963.
4. Harter, J. G.: The Effect of Storage on Human Skin-Sensitizing Antibodies (Reagin), *Fed. Proc.* **20**: 259, 1961.
5. Perlman, F., and Wolfe, H. F.: Dimethyl Sulfoxide as a Penetrant-Carrier of Allergens Through Intact Human Skin, *J. ALLERGY* **38**: 299, 1966.
6. Layton, L. L., Yamanaka, E., and Denko, C. W.: Demonstration of Human Reagins to Foods, Cat Dander, An Insect, and Ragweed and Grass Pollens, *J. ALLERGY* **33**: 271, 1962.
7. Pruzansky, J. J., Feinberg, S. M., Haminker, A., and Meleyco, L. W.: Antigenic Relationship of Insect Extracts, *J. ALLERGY* **30**: 332, 1959.