Reproducible Passive Hemagglutination Procedure with High Sensitivity Using the Double Aldehyde Treated Cells (33552)

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Previously, we reported on a passive hemagglutination procedure in which antigen-coated erythrocytes could be stored frozen or in a lypophilized state (1). Erythrocytes were stabilized by pyruvic aldehyde followed by formaldehyde. It was also pointed out that the passive hemagglutination (HA) titers differed considerably when different cell preparations were used, presumably due to biological variability of native erythrocytes.

It was found that by subjecting antigen-coated cells to an appropriate freezing and thawing schedule, such cells would yield highly sensitive HA titers. Reproducibility was ensured by storing antigen-coated cells in liquid nitrogen.

Materials and Methods. Stabilized rabbit erythrocytes were coated with bovine serum albumin (BSA) as previously described (1). In brief, erythrocytes were stabilized by exposure to pyruvic aldehyde followed by formaldehyde and washed. The stabilized erythrocytes were designated as FPRE. Packed FPRE (0.1 ml) were resuspended in 10 ml of 0.1 *M* acetate buffer, pH 4.0, containing 1 mg of BSA and stirred for 2 hr (BSA-FPRE). Antigen-coated cells were washed 5 times with 0.11 *M* phosphate buffer, pH 7.2, and resuspended as 10% in the phosphate buffer. The coated cells were quick frozen in liquid nitrogen and stored either at —20° or in liquid nitrogen (—196°).

Hyperimmune rabbit anti-BSA sera were used. Antisera were diluted in 0.11 *M* phosphate buffer containing 1 mg of gelatin/ml (Gel) (Bactogelatin, Difco Laboratories, Detroit, Michigan).

The HA reaction was performed on plastic trays with 96 multiple "V" bottom wells (Cooke Engineering Co., Alexandria, Virginia, Catalog 220-25 "V" plate, disposable, or Linbro Chemical Co., New Haven, Connecticut, Cat. IS-MVC-96, disposable).

The HA test was performed by adding 25 μl of 0.25% coated FPRE to 25 μl of serial 2-fold dilutions of antiserum. The trays were shaken three times at 10-min intervals and allowed to stand at room temperature on a vibration-free lead plate. The HA patterns were read 18 hr later.

Effect of freeze storage of Ag-coated cells on HA titers. Although we previously reported that antigen-coated FPRE could be stored frozen without appreciable change in HA titer (1), a close examination disclosed otherwise.

When coated cells were stored at —20°, periodically thawed and used for HA titrations, the titers were found to become higher with increase in storage time; during a course of a 4-month period, as much as 30-fold increase was encountered (Table I).

The rate of increase in HA titers with freeze-storage of BSA-FPRE differed considerably from one cell preparation to another. Thus, an attempt was made to control this variation.

Abolition of differences in HA reactions due to biological variations of cell preparations. It was found that the increase in HA titers with freeze-storage of BSA-FPRE could be accelerated by repeated freezing and thawing. Coated cells were frozen at —196° and stored at —20°. They were allowed to thaw at room temperature (24°) without shaking for 2 hr, refrozen at —196° and then stored at —20°. This process was repeated once a week. After repeated freezing and thawing for 6 times, the titer rose 30-fold (Table I). The titer for the normal serum control (preimmunization serum) remained at 1/4. Further repeated freezing and thawing procedure resulted in increased HA

TABLE I. Effect of Repeated Freezing and Thawing of Antigen-Coated Cells on HA Titers.

	HA titer ($\times 10^{-4}$), reciprocal; duration of storage (weeks)				
Conditions of BSA-FPRE storage	0	2	4	6	16
—20° with freeze/thaw[a]	4	8	32	128	—
—20° without freeze/thaw	4	8	—	32	128
—196° without freeze/thaw	4	—	—	8	8
—196° after optimal condition attained by freeze/thaw six times	128	—	—	—	128

[a] Freezing and thawing once per week.

titers but so did the nonspecific agglutination titers with the control serum. With other coated cell preparations, the freezing and thawing process had to be repeated 8 or 9 times before the optimal cell preparations were obtained. The optimal cell preparation is defined here as a preparation which gave a maximum HA titer with an arbitrarily selected antiserum (1,280,000 for this serum) without appreciable nonspecific agglutination titer in normal serum (1/4 to 1/8). A coated cell preparation which was optimal for the standard serum was optimal also for other antisera. Thus successive standard antisera can be standardized against the originally selected serum.

Once the optimal condition was achieved, the coated cells were freeze-stored at —196°; HA titer did not change for at least 16 weeks when stored at this temperature.

As a routine we now freeze and thaw antigen-coated FPRE once a week until the HA titer reaches 1.28×10^{6} with a standard antiserum, then store the cells in liquid nitrogen.

With some hyperimmune sera, containing 14–16 mg of antibody/ml, titers of $6\text{–}8 \times 10^{6}$ were obtained.

Discussion. In our previous publication we reported that when several batches of FPRE were prepared from different rabbits, the passive HA titers differed as much as 30-fold (1). In order to abolish this variability, attempts were made to ascertain the conditions necessary to obtain cell preparations which yield high titers. Our initial efforts were focused on obtaining erythrocytes with uniform characteristics. To this end we compared cells collected at various times of the year, altered the volume of Alsever's solution as related to blood volume, studied the effect of the length of time between blood collection and aldehyde treatment, compared cells obtained from venous and arterial blood, and evaluated erythrocytes exposed to hypotonic solutions prior to aldehyde treatment. BSA-FPRE prepared from the above erythrocyte preparations, however, gave low passive HA titers (10,000–40,000).

The pyruvic aldehyde, used for stabilization of cells, was purified by vacuum distillation. FPRE prepared with various concentrations of purified pyruvic aldehyde and formaldehyde also yielded low titers.

We also reported that antigen-coated FPRE could be stored frozen at —20° without apparent alterations (1). Upon close examination, however, it was found that coated cells do change during freeze-storage. The HA titer was higher with the use of coated cells freeze-stored for a prolonged period than with those frozen for a short duration.

This increase in titer was accelerated by repeated freezing and thawing once a week for several weeks. An attempt to shorten this period by freezing and thawing thrice weekly was not fruitful.

After the desired titer was obtained, the coated cells were stored at —196°. At this temperature the increase in titer was minimal (Table I).

Summary. The sensitivity of the passive hemagglutination reaction was increased considerably by repeated freezing and thawing of antigen-coated erythrocytes. Cells were stabilized by pyruvic aldehyde followed by

formaldehyde and then coated with antigen at low pH. After the desired HA titer was attained, the cells were stored in liquid nitrogen. Antigen-coated cells stored at —20° gradually deteriorated during the course of 18 months.

1. Hirata, A. A. and Brandriss, M. W., J. Immunol. 100, 641 (1968).

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A Comparative Study of the Leukemogenic Effects of Strontium-90 and X-Rays in Mice (33553)

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Radiostrontium (^{85}Sr, ^{89}Sr, and ^{90}Sr) is selectively deposited in the skeleton and induces osteogenic sarcomas (1). Finkel (2) reported that it can also induce leukemia, although the incidence is low. While studying the induction of osteogenic sarcomas by ^{90}Sr, we observed a high incidence of leukemia in a low leukemia strain of mice. In the present paper, the leukemogenic effect of ^{90}Sr is compared with that of fractionated total-body X-irradiation.

Materials and Methods. Animals. Female mice of ICR/JCL strain, purchased from the Nihon Clea Co., Tokyo, Japan (CLEA), were employed. This strain stems from ICR/Ha, in which incidence of spontaneous leukemia is less than 1.0% (3). Mice were kept in metal cages, 10 animals in each, in an air-conditioned animal room and maintained on commercial pellets and water *ad libitum.* Thirty-three ICR/JCL female mice, of the line originally derived from CLEA and subsequently bred in this laboratory by brother-sister mating, have been maintained without any treatments as controls.

^{90}Strontium. ^{90}Strontium chloride ($^{90}SrCl_2$), purchased from the Radiochemical Center, Amersham, Buckinghamshire, England, was diluted with physiological saline.

X-irradiation: X-irradiation was provided with a 180 kVp X-ray generator, the factors being 25 mA, HVL 1.18 mm Cu, with 0.5 mm Cu and 0.5 mm Al filters; it delivered at 50 rads/min at the TSD 65 cm.

Expt. I. The mice were divided into 4 groups at random. The first group was injected intraperitoneally with 1.0 μCi of ^{90}Sr/g of body weight at 5 weeks of age. The second group was thymectomized at 3 weeks of age and injected with 1.0 μCi/g of ^{90}Sr at 5 weeks of age as the first group. The third group was X-irradiated with 1000 R at 4 weeks of age over the right hind leg while other parts of the body were shielded with 10-mm thick lead plates, and was injected with 1.0 μCi/g of ^{90}Sr at 5 weeks of age. The fourth group was given only 1000 R over the right hind leg as the third group. Expt. I was started with 63 mice, 17 in the first, 14 in the second, 16 in the third, and 16 in the fourth group.

Expt. II. Expt. II was started 6 months after Expt. I. The mice were divided into 4 groups at random. The first two groups were treated in the same way as the first or the second group in Expt. I. The third group was given total-body irradiation in four doses of 170 R X-rays at 5-day intervals beginning at 4 weeks of age, according to the schedule of Kaplan and Brown (4). The fourth group was previously thymectomized at 3 weeks of age and given total-body irradiation of the same doses of X-rays as the third group. Expt. II was started with 83 mice, 17 in the first, 20 in the second, 22 in the third, and 24 in the fourth group.

The mice were sacrificed when moribund or showing typical symptoms of leukemia or osteogenic sarcoma. All animals were autopsied. Sections for microscopic examination