Enhanced Production of Antibody to a Murine Leukemia Virus (Rauscher) in the Host of Origin (32785)

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The BALB/c mouse, from which several murine leukemia virus strains originated, has been used extensively for immunological studies in murine leukemia. These studies have shown that this natural host can be actively immunized by the inoculation of BALB/c derived, formalin-treated virus. The serums of mice immunized with vaccine emulsified in Freund's adjuvant contain antiviral antibodies demonstrable by passive immunization, neutralization, and by several *in vitro* serological tests (1).

When serological procedures were being developed for the detection and identification of strains of murine leukemia virus, the need for a strong precipitating antibody produced in the syngeneic host became evident. A protocol known to produce active immunity to Rauscher virus, one in which animals were inoculated on the first day with formalin-treated virus plus complete Freund's adjuvant, followed in 28 days by a booster inoculation, provided only weakly reactive serum. In an attempt to increase the antibody concentration, two experiments involving the use of immunological adjuvants were performed. In the first experiment, the original protocol using Freund's adjuvant was modified to test the effect of increasing the interval between the primary and secondary inoculation. In the second experiment, the adjuvant effect of bacterial lipopolysaccharide (endotoxin) on a primary injection of formalin-treated virus was studied. This report presents the results of these two approaches, including the kinetics of the primary and secondary antibody responses to this virus as well as some characteristics of the antibody produced by the different methods.

Materials and Methods. Virus. For vaccine preparation, Rauscher virus from 10% cell-free extracts of infected BALB/c spleen was treated with formaldehyde to a final concentration of 0.1% and stored for at least 2 weeks at 0–4°C before use (2). Spleen extracts from normal mice were prepared in the same manner. For serological tests, virus from infected BALB/c plasma was concentrated and purified by zonal ultracentrifugation on a potassium citrate gradient. The 1.16 density isolate used in these studies was prepared by Mr. I. Toplin at Charles Pfizer and Co., under contract to the National Cancer Institute (3).

Endotoxin. Bacterial lipopolysaccharide or endotoxin (ET), extracted from *S. abortus equi* by the Westphal method, was obtained from the Difco Laboratories, Detroit, Michigan (Control no. 169570). The LD_{50} of this lot for BALB/c mice was 10 mg/kg.

Treatment of mice. In one experiment, BALB/c mice of both sexes, at least 10 weeks of age, were injected intraperitoneally on day 1 with 0.25 ml of vaccine emulsified with 0.25 ml of complete Freund's adjuvant, as before (1). These mice were then divided into five groups with care being taken to minimize any variability due to sex and age by equal distribution of available mice throughout the various groups. The groups were then treated as follows: half of the mice in each group were left untreated, the other half were given a subcutaneous booster inoculation of 0.10 ml of vaccine without adjuvant on day 30, 60, 75, 90, or 126. On the day indicated, the half receiving only a primary inoculation were bled; the other half were given a booster inoculation, and bled seven days later. Approximately equal amounts of serum from each of the 10 mice treated in the same way were pooled. The physical pooling of serums has been shown to give reliable results when compared to the average of individual runs (4). The serums from each group were stored at —20°C until all were collected.

In another experiment, male BALB/c mice, 8–10 weeks old, were inoculated intraperi-

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toneally with 0.25 ml of the following materials: (a) 25 μg of ET, (b) vaccine, (c) vaccine plus 25 μg of ET, or (d) formalin-treated spleen extract from normal mice plus 25 μg of ET. Animals were bled at various times after inoculation and individual serums were stored at −20°C until tested.

Antibody determinations. The serums were tested by the following techniques: *A.* Immunodiffusion, in which the serums were tested against Rauscher virus using a microtechnique as previously described (5). Briefly, this consisted of the addition of the reactants into the wells of a plastic template superimposed on 0.75% agarose on a standard microscopic slide. After 48 hours at room temperature, the templates were removed and the tests read. *B.* Passive hemagglutination (HA), previously used with Rhesus monkey antiserum (6), was adapted for testing mouse serums. Rauscher virus at a concentration of 50 μg of protein/ml, representing four times that used for monkeys, was found to be optimal for measuring both gamma-G and gamma-M antibodies and was the only modification in the preparation of sensitized sheep erythrocytes (SRBC). Serums inactivated at 56°C for 30 min and absorbed to completion with packed SRBC were serially diluted in 0.3 ml volumes, and 0.03 ml of 2.5% sensitized SRBC were added to each tube. After 3 hours at room temperature, the tests were read and the reciprocal of the dilution showing complete HA was taken as the end point of the titration. All appropriate controls were included in each test. *C.* Neutralization, in which various dilutions of virus were mixed with the standard dilution of antiserum were performed under the direction of Dr. G. Spahn at the Microbiological Associates, Walkersville, Maryland, under contract to the National Cancer Institute. After incubation of the virus–antiserum mixture at room temperature, groups of mice were inoculated with it, then observed for 120 days. Results are recorded as the neutralization index (NI) which represents the log of virus neutralized under the standard conditions of the test. A detailed description of this method has been published elsewhere (1).

Sucrose gradient analysis. Sedimentation properties of antibodies were determined by zonal density gradient ultracentrifugation in a Spinco Model L centrifuge employing a SW-39 rotor and sucrose gradients from 10 to 37% (7). After centrifugation at 100,000*g* for 18 hours, 10 fractions were obtained by collecting successive drops from the bottom of the tube.

TABLE I. Effect on Antibody Titer of Interval between Primary and Secondary Inoculation.

Group	Interval (days)	Hemagglutination	Immunodiffusion	Neutralization index
I-P[a]	30	20	—	0.5
-S[b]		40	—	0.8
II-P	60	40	—	0.5
-S		80	±	0.6
III-P	75	80	±	0.9
-S		160	+	1.2
IV-P	90	40	—	1.2
-S		320	++	1.3
V-P	126	20	—	
-S		320	++	

[a] Primary antigen stimulus.
[b] Secondary antigen stimulus.

Results. The first series of experiments were performed to determine whether an increase in the interval between a primary injection of vaccine emulsified in Freund's adjuvant and a second inoculation would affect the antibody response to Rauscher virus. The results presented in Table I show that in the absence of a booster inoculation, HA activity reached its peak on day 75 and by day 126 had dropped to its original 30-day level. The only demonstrable precipitating antibody, and that very weak, occurred on day 75. After a second inoculation, the HA titer rose steadily to day 90 where it remained until day 126, the last day of testing. Precipitating antibody was detectable as the interval between the primary and secondary inoculation increased from day 75. The data from Table I and Fig. 1 clearly indicate that the secondary response was maximal only after the primary response had begun to wane. The results of neutralization tests are also given in Table I. Little neutralizing antibody was demonstrated until day 75 where a significant amount was detected both before and after the booster.

TABLE II. Reciprocal of Geometric Mean Titers of Mice Showing HA Activity to Rauscher Leukemia Virus.

Treatment	Days after injection			
	5	8	11	14
ET (25 μg)	<5 (0/7)[a]	<5 (0/6)	<5 (0/8)	<5 (0/7)
Vaccine (0.25 ml)	<5 (0/7)	<5 (0/6)	<5 (0/8)	<5 (0/8)
Vaccine (0.25 ml) plus ET (25 μg)	80 (5/6)	540 (6/6)	8 (2/8)	<5 (0/7)

[a] Figures in parentheses indicate no. positive/no. tested.

The second experiment was carried out to determine whether endotoxin affects the antibody response of the mouse to Rauscher leukemia virus. The serums of mice inoculated with vaccine plus ET showed HA activity which was detected as early as day 5 (Table II). In contrast, mice given ET alone or vaccine alone failed to respond. An additional group of ET-stimulated mice given formalin-treated spleen extract from normal mice showed no HA activity to Rauscher virus.

The antibody response of ET-stimulated mice to Rauscher virus vaccine was further investigated. Figure 2 shows that serum HA activity appeared on day 3, and reached maximal levels by day 7 or 8. Forty-four of 47 serums collected from day 3 through day 10 showed HA activity. Representative serums, all of which showed measurable HA activity, failed to precipitate with virus when control serum obtained after immunization with virus emulsified in Freund's adjuvant always reacted.

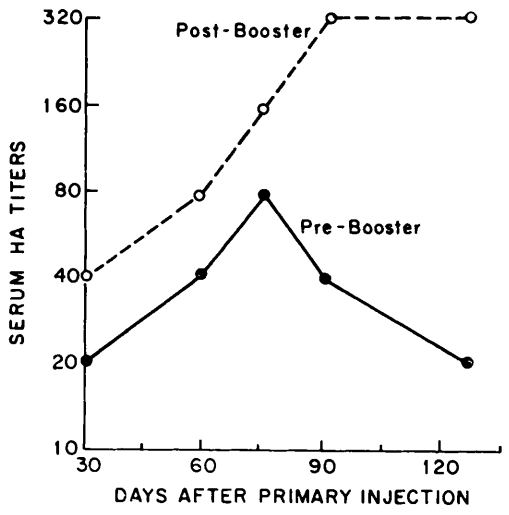

FIG. 1. Antibody titer following booster injections given at various intervals. Each point represents the pooled serums of 10 mice.

Sucrose density gradient analysis of pooled mouse serums taken 4, 6, and 9 days after administration of vaccine plus endotoxin showed that HA activity was restricted to the gamma-M globulin fractions (Table III).

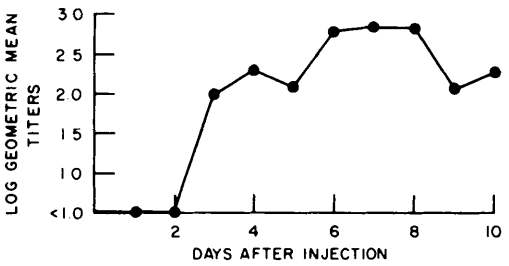

FIG. 2. Antibody response of mice inoculated with Rauscher virus vaccine plus endotoxin. Each point represents the geometric mean of a minimum of 5 individual mice.

Discussion. The BALB/c mouse was chosen for the production of antibody to Rauscher virus because the virus used for immunization was derived from this strain. In earlier work, a standard regimen (i.e., a 28-day interval between inoculation and challenge or inoculation and booster) yielded mice which were solidly immune to leukemia either before or after a booster inoculation (1). Although the serum of these animals contained antibodies demonstrable by various serological tests, the amount of antibody was less than desirable for use as a diagnostic reagent for immunoprecipitation studies.

By increasing the interval between primary and secondary stimulation, we have prepared high-titer antiserum for use in serological tests for a murine leukemia virus (Rauscher). Our findings indicate that the antibody response

TABLE III. HA Activity of Sucrose Density Gradient Fractions from Pooled Serums of 10 Mice Given Vaccine plus Endotoxin.

Fraction no.[a]	Reciprocal of HA titers		
	Day 4	Day 6	Day 9
1	80	40	80
2	160	80	80
3	<10	10	10
4–10	<10	<10	<10
Whole serum	320	320	80

[a] Fractions 1–3 were taken from the bottom of the centrifuge tube; fractions 4–10 were tested individually.

to secondary inoculation becomes greater after the primary response has begun to wane. This observation apparently does not hold true for the neutralizing antibody which was relatively unaffected by the booster inoculation, and suggests that the two serological techniques are probably more sensitive or may not be measuring the same antibody as neutralization tests. Other studies bearing on the effect of length of interval between primary and secondary antigenic stimuli in mice include the work of Fescik *et al.* (4). These workers demonstrated an increase in titer to diphtheria toxin as the interval between injections was lengthened up to 40 days, but not beyond. The difference in results between those reported here and those of Fescik *et al.* are probably due to the use of Freund's adjuvant in the present studies, instead of fluid antigen.

Enhancement of antibody formation in mice by endotoxin has been amply confirmed (8). Mice given vaccine plus ET showed HA activity in the serums 3 days after a primary injection. There was no HA activity in the serums when either vaccine or ET was given alone. This finding enlarges the previous work of Fink and Rauscher (2) who showed that vaccine given to mice in the absence of Freund's adjuvant was, in comparison to the same amount of vaccine given with an adjuvant, relatively ineffective in stimulating the production of circulating antibodies to the Rauscher virus as measured by neutralization and virus challenge. HA activity during the ET-stimulated antibody response was restricted to the gamma-M component. Studies conducted in various animal species, including inbred mice, have shown that following a single injection of virus antigen, the early primary response consisted mainly of gamma-M antibody (7, 9–11). Only in the later phases of the primary response was the gamma-G component demonstrated and its appearance was dependent on the dose employed (9, 11). Since preliminary experiments using HA tests established that the virus concentration used to prepare sensitized SRBC readily detected gamma-M and gamma-G antibodies to this virus, it is unlikely that this method of testing would have failed to detect activity in the gamma-G component if it were present.

Although they had significant HA titers, serums collected during the primary response usually did not react with virus in immunodiffusion tests. This finding parallels our earlier results with gamma-M antibody from Rhesus monkeys immunized with Rauscher virus (6). Since the failure of serums to precipitate could not entirely be attributed to low antibody concentrations, other explanations were considered. One possibility is that gamma-M antibodies which predominate during the early response do not precipitate with virus. Other investigators have shown that gamma-M antibodies to protein antigens were not detectable in gel diffusion tests even though HA titers were high (12, 13). Another possibility suggested by the work of Goodman and Masaitis (14) is that early antiserums may be comprised of antibodies specific for a single, nonrepeating determinant group of the antigen. Such serum would be incapable of producing a lattice formation, but would react in HA tests where many antigen molecules are coupled to tanned cells. Our experiments, therefore, point to the value of the combined use of precipitation and HA for determining antibody activity to the Rauscher virus.

Summary. Potent antiserum was prepared against a murine leukemia virus (Rauscher) in BALB/c mice by an immunizing regimen consisting of two inoculations in which the second one is given only after an interval sufficient for the primary response to wane. An interval of 90 days or more following a primary injection of antigen emulsified in Freund's adjuvant was optimal. The early

antibody response to Rauscher virus in ET-treated mice is also described. The gamma-M antibodies, which appeared as early as the third day after a single antigenic stimulus, were readily detected by HA tests, but failed to precipitate with virus in immunodiffusion tests.

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1. Fink, M. A., Rauscher, F. J., and Chirigos, M., in "Viruses Inducing Cancer," Burdette, W. J., ed., p. 25. Univ. of Utah Press, Salt Lake City, Utah, 1966.

2. Fink, M. A. and Rauscher, F. J., J. Natl. Cancer Inst. **32**, 1075 (1964).

3. Toplin, I., Appl. Microbiol. **15**, 582 (1967).

4. Fescik, A. I., Butler, W. T., and Coons, A. H., J. Exptl. Med. **120**, 1041 (1964).

5. Fink, M. A. and Cowles, C. A., Science **150**, 1723 (1965).

6. Sibal, L. R., Fink, M. A., McCune, C. L., and Cowles, C. A., J. Immunol. **98**, 368 (1967).

7. Svehag, S. and Mandel, B., J. Exptl. Med. **119**, 1 (1964).

8. Munoz, J., "Advances in Immunology," Dixon, F. J. and Humphrey, J. H., eds., Vol. 4, p. 397. Academic Press, New York, 1964.

9. Svehag, S. and Mandel, B., J. Exptl. Med. **119**, 21 (1964).

10. Berlin, B. S., J. Immunol. **96**, 425 (1966).

11. Lefkowitz, S. S., Williams, J. A., Howard, B. E., and Sigel, M. M., J. Bacteriol. **91**, 205 (1966).

12. Möller, G., J. Immunol. **96**, 430 (1966).

13. Shulman, S., Hubler, L., and Witebsky, E., Science **145**, 815 (1964).

14. Goodman, H. S. and Masaitis, L., Nature **215**, 392 (1967).

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Effect of pH on Active Transport of d-Glucose in the Small Intestine of Hamsters* (32786)

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Laszt(1) first noted that the absorption of glucose was dependent upon pH. While several studies on the effect of pH on glucose absorption have been reported over the years, the topic is still controversial. Groen(2) observed maximal glucose absorption in man at a pH of 7.0 while Goldenberg and Cummins (3) noted that glucose absorption was greater at alkaline pH than at acid pH. Ponz and Larralde (4), in the isolated jejunal loop of rats, found a plateau of optimal glucose absorption between pH 6.5 to 7.5. Broitman *et al.* (5) intubated intact rats and observed that glucose absorption increased as the pH was increased from 6.3 to 7.0.

A major difficulty in ascertaining an optimal pH for glucose absorption either in the intact animal or in isolated loops is the intraluminal pH adjustment by the small bowel. It is well recognized that in both man and animals, solutions introduced into the intestine undergo a change in pH towards neutrality, owing to the buffer capacity of intestinal secretions (3–7). The present study was therefore conducted using everted sacs in which the pH of the mucosal bathing solution could be maintained constant.

Material and Methods. Male golden hamsters weighing 84–120 gm were fasted for 24–48 hours, then killed by a blow on the head, decapitated and their blood drained. The sacs were immediately prepared as described by Wilson(8). Six to seven intestinal

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