PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **147**, 242–244 (1974)

Effect of Delayed Hypersensitivity on Plasma Iron and Zinc Concentration and Blood Leukocytes (38318)

RALPH F. KAMPSCHMIDT AND LARRY A. PULLIAM

Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma 73401

Delayed hypersensitivity has been shown by Uhr and Brandriss (1) to cause a febrile response accompanied by lymphopenia in guinea pigs. These studies were expanded by Atkins *et al.* (2, 3) to show that sensitized lymphocytes did not release endogenous pyrogen, but did release a soluble agent which activated peripheral blood neutrophils or monocytes to synthesize endogenous pyrogen. Our recent investigations have indicated that endogenous pyrogen and leukocytic endogenous mediator (LEM) are closely related proteins (4). In addition to its pyrogenic activity, LEM when injected also will cause lowering of plasma iron (5) and zinc (6) concentrations and the release of neutrophils from bone marrow to the peripheral blood (7). If the febrile response during delayed hypersensitivity was due to the synthesis of endogenous pyrogen (3), it seemed likely that LEM was also being produced. Delayed hypersensitivity should then have a pronounced effect on peripheral blood iron and zinc concentrations and the number of neutrophils.

Materials and Methods. Animals. Female Holtzman rats 7–10 weeks of age and weighing 160–200 g were maintained at 22° with 12 hr of light and 12 hr of darkness and fed Rockland rat diet and water *ad libitum*. Rabbits were New Zealand white and were purchased locally.

Producing and testing for delayed hypersensitivity. Bovine serum albumin (BSA) (Sigma A-4378) was dissolved in distilled water and emulsified at a 1:1 ratio with Freund's complete adjuvant (Difco 311-59). Rabbits received a total dose of 2 ml of this emulsion: 0.2 ml in each front foot pad; 0.5 ml in the rear foot pads; and 0.6 ml in four different subcutaneous sites. A total of 0.1 ml of the emulsion was injected into one hind foot pad of each rat. Fourteen days later some rats and/or rabbits from each group were given intradermal injections of BSA (100 μg in 0.1 ml of 0.9% NaCl). They were examined for edema 24 hr later. Challenging doses of BSA were injected 14–18 days after sensitization.

Measurement of biological activities. The body temperature of rabbits was monitored using rectal thermisters (Yellow Springs Instrument Co., Yellow Springs, OH) which were connected to a telethermometer. After establishing a base line we injected the test material iv and recorded the temperature every 15 min.

Plasma iron, plasma zinc, and total blood neutrophils were measured 8 hr after an ip injection of the test dose in rats and after an iv injection in rabbits. Blood was collected from the heart of anesthetized rats and from an ear vein of unanesthetized rabbits. The plasma iron concentration was determined by the 2,2′,2″-terpyridine method (8). Plasma zinc was measured with a model 403 Perkin–Elmer atomic absorption spectrophotometer after diluting the plasma with 3 volumes of water. Total blood neutrophils were determined by diluting the blood 1-200 with Turk's diluting fluid and counting total leukocytes in a hemocytometer, followed by a 200-cell differential count of a smear stained with Wright's stain.

Results. Rabbits which were sensitized to 10 mg of BSA and challenged 2 weeks later with 0.5 mg BSA had an average increase in body temperature of 1.5°. The fever developed slowly and was still well above normal at 6 hr. Unsensitized rabbits injected with 0.5 mg of BSA showed no significant change in body temperature. The effects of a challenge with BSA on the concentrations of plasma iron and zinc as well as total blood neutrophils are presented in Table I. The control groups were rabbits which had received the sensitizing dose but not the challenge dose and rabbits which had received only the challenge dose. When the rabbits were given both the sensitizing and challenging doses of

TABLE I. Effect of Delayed Hypersensitivity on Plasma Iron Concentration, Plasma Zinc Concentration and Number of Peripheral Blood Leukocytes in Rabbits.[a]

No. of rabbits	Sensitization (mg BSA[b])	Challenge (mg BSA[b])	Plasma iron (μg%)	Plasma zinc (μg%)	Circulating leukocytes (per mm^3 × 10^{-2})		
					Total	Neutrophils	Lymphocytes
19	None	None	174 ± 8[c]	247 ± 7	75 ± 4	17 ± 2	55 ± 3
9	10	None	144 ± 6	233 ± 7	73 ± 8	23 ± 4	47 ± 6
11	None	0.5	150 ± 12	243 ± 8	65 ± 5	20 ± 4	44 ± 4
12	10	0.5	83 ± 4[d]	123 ± 10[d]	127 ± 5[d]	107 ± 5[d]	18 ± 2[d]

[a] Rabbits were sensitized by injecting an emulsion containing 10 mg BSA and Freund's complete adjuvant. They were challenged 14–18 days later with BSA in 0.9% NaCl. See Methods.

[b] Bovine serum albumin. Dose per rabbit.

[c] Mean ± SE.

[d] Significantly different from any of the 3 control groups: $P < 0.01$.

BSA, plasma iron and plasma zinc concentrations decreased 50%. Total blood neutrophils were increased over fivefold but peripheral blood lymphocytes showed a marked decrease in number. When LEM was injected in rabbits, there was a pronounced increase in blood neutrophils but very little effect on the number of lymphocytes.

A similar experiment was conducted in rats, and these results are shown in Table II. Neither the sensitizing nor the challenging dose of BSA alone significantly effected any of the biological parameters measured. When the rats were sensitized and 2 weeks later challenged with BSA, plasma iron and plasma zinc concentrations were significantly lowered. Peripheral blood neutrophils were increased five- to sixfold, but lymphocytes were not markedly depressed. Varying the sensitizing dose of BSA between 0.1 and 5.0 mg/rat did not alter the results. However, when the challenge dose of BSA was varied, there seemed to be a direct relationship between dose and response.

Discussion. Previous results have suggested several similarities between endogenous pyrogen and LEM; they can be isolated from the same source (5, 9) and they share in common several physical and chemical properties (4). It should be noted, however, that some differences in these properties and in species specificity have been reported (10). The present results suggest that proteins causing both of these types of effects, *i.e.*, fever and the biological alterations attributed to LEM, are released during delayed hypersensitivity.

The experiments of Atkins and Francis (2)

TABLE II. Effect of Sensitization and Challenge with Bovine Serum Albumin on Plasma Iron Concentration, Plasma Zinc Concentration and Number of Peripheral Blood Neutrophils in Rats.[a]

No. of rats	Sensitization (mg BSA[b])	Challenge (mg BSA[b])	Plasma iron (μg%)	Plasma zinc (μg%)	Circulating leukocytes (per mm^3 × 10^{-2})		
					Total	Neutrophils	Lymphocytes
28	None	None	241 ± 9[c]	103 ± 4	96 ± 3	13 ± 2	77 ± 4
18	0.1–5.0[d]	None	255 ± 15	95 ± 5	99 ± 3	13 ± 2	83 ± 5
28	None	0.5	244 ± 14	101 ± 4	100 ± 4	15 ± 2	86 ± 4
28	0.1–5.0[e]	0.5	116 ± 8[f]	48 ± 4[f]	156 ± 6[f]	83 ± 6[f]	69 ± 4

[a] See the Methods section for details of the experimental technique.

[b] Bovine serum albumin dose per rat.

[c] Mean ± SE.

[d] Three groups of six rats each received 0.1 mg, 2.0 mg, and 5.0 mg, respectively. Since no difference was observed the groups were combined.

[e] Three groups of six rats each received 0.1 mg, 2.0 mg, and 5.0 mg, respectively. One group of ten rats received 0.5 mg. Since no differences were found the 4 groups were combined in this table.

[f] Significantly different from any of the three control groups: $P < 0.01$.

indicated that properly stimulated and challenged lymphocytes released a soluble agent which then activated phagocytic cells to synthesize endogenous pyrogen. The present investigation suggests that this soluble agent from lymphocytes also may be responsible for the synthesis of LEM by phagocytic leukocytes. The metabolic alterations observed following delayed hypersensitivity were similar to those found after injecting LEM (4). The one exception was the effect on the number of peripheral blood lymphocytes. Injection of LEM in rats (7) or rabbits produced a several fold increase in the number of peripheral blood neutrophils but only a slight effect on the number of lymphocytes. Delayed hypersensitivity caused a marked lymphopenia in guinea pigs (1) and rabbits but not in rats. Apparently the effect on the number of peripheral blood lymphocytes during delayed hypersensitivity was not mediated by LEM.

Fevers that developed slowly after challenging sensitized rabbits were seen previously by Atkins *et al.* (3) who suggested that this may help explain fevers associated with inflammations not caused by infection. Thus, endogenous pyrogen can be produced by phagocytic leukocytes following engulfment of exogenous materials, such as bacteria or viruses, or by soluble agents from sensitized lymphocytes (3). Challenging sensitized rats or rabbits apparently also causes the production of LEM. This may assist in clarifying why plasma iron and zinc concentrations are lowered during trauma or neoplastic growth when exogenous agents apparently are not involved (11–14).

Summary. Rats and rabbits were sensitized to bovine serum albumin by injecting it in an emulsion with complete Freund's adjuvant. Two weeks later these animals were rechallenged with bovine serum albumin which produced a decrease in plasma iron concentration, plasma zinc concentrations, and a marked increase in peripheral blood neutrophils. The possibility that soluble agents from stimulated lymphocytes activated phagocytic cells to synthesize leukocytic endogenous mediator during delayed hypersensitivity was discussed.

We acknowledge with thanks the skillful technical assistance of Faye Jones.

1. Uhr, J. W., and Brandriss, M. W., J. Exp. Med. **108,** 905 (1958).

2. Atkins, E., and Francis, L., J. Infect. Dis. **128,** 5277 (1973).

3. Atkins, E., Feldman, J. D., Francis, L., and Hursch, E., J. Exp. Med. **135,** 1113 (1972).

4. Kampschmidt, R. F., Upchurch, H. F., Eddington, C. L., and Pulliam, L. A., Amer. J. Physiol. **224,** 530 (1973).

5. Kampschmidt, R. F., and Upchurch, H. F., Amer. J. Physiol. **216,** 1287 (1969).

6. Kampschmidt, R. F., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **134,** 1150 (1970).

7. Kampschmidt, R. F., Long, R. D., and Upchurch, H. F., Proc. Soc. Exp. Biol. Med. **139,** 1224 (1972).

8. Schade, A. L., Oyama, J., Reinhart, R. W., and Milton, J. R., Proc. Soc. Exp. Biol. Med. **87,** 443 (1954).

9. Kampschmidt, R. F., Upchurch, H. F., and Pulliam, L. A., Proc. Soc. Exp. Biol. Med. **143,** 279 (1973).

10. Pekarek, R. S., Wannemacher, R. W., Jr., Chapple, F. E., III, Powanda, M. C., and Beisel, W. R., Proc. Soc. Exp. Biol. Med. **141,** 643 (1972).

11. Syrkis, I., and Machtey, I., J. Amer. Geriatrics Soc. **21,** 28 (1973).

12. Lindeman, R. D., Bottomley, R. G., Cornelison, R. L., Jr., and Jacobs, L. A., J. Lab. Clin. Med. **79,** 452 (1972).

13. Rosner, F., and Gorfien, P. C., J. Lab. Clin. Med. **72,** 213 (1968).

14. Griffin, A. C., Dale, S. C., Wellington, D., and Word, V. C., Proc. Soc. Exp. Biol. Med. **118,** 741 (1965).

Received February 20, 1974. P.S.E.B.M., 1974, Vol. 147.