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Serum Calcium and Hepatic Kupffer Cell Phagocytosis[1] (38764)

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Previous studies from this laboratory (1–4) and others (5, 6) have resulted in the development of an *in vitro* isotopic tissue slice bioassay for the determination of opsonic phagocytosis promoting activity of animal and human serum. With the use of this technique, it has been demonstrated that opsonic dysfunction exists following starvation, R.E. blockade, and whole-body trauma as well as during the terminal stage of tumor growth (1, 3, 5) and that the administration of opsonic protein can lead to R.E. activation and decreased tumor growth (4, 6). These findings coupled with the well-documented role of the phagocytic or macrophage system in anti-bacterial and anti-tumor defense have emphasized the need to clarify the factors that can influence the opsonin mediated stimulation of *in vitro* phagocytosis as monitored by the liver slice bioassay. Moreover, since alterations in nonopsonic factors in serum could influence the phagocytosis promoting activity of serum an understanding of these factors was considered crucial to the comphrehension of the changes in *in vivo* reticuloendothelial (R.E.) phagocytosis that exist following injury and tumor transplantation (1, 2).

Previous analysis of serum ionic composition with experimental (7–9) and clinically encountered (10–12, 15–17) neoplasms, with and without bone metastases, have reported a frequent pronounced hypercalcemia during tumor growth. Upon medically induced regression or surgical resection of these tumors the calcium levels have been observed to return to normal levels, while tumor recurrence is accompanied by a reappearance of hypercalcemia (10, 11). Recent observations from this laboratory on the functional state of the R.E.S. during tumor growth have revealed that the terminal stages of tumor growth and spread are associated with pronounced serum opsonic dysfunction and associated depression in hepatic Kupffer cell phagocytosis (18). These findings when placed in relationship to the temporal change of calcium levels with tumor growth suggest a potential effect of serum calcium concentration alterations on reticuloendothelial function. In the present study the effect of defined experimental alterations of serum calcium levels on opsonin mediated hepatic phagocytosis was investigated.

Materials and Methods. Male Holtzman (Holtzman Co., Madison, WI) rats weighing 250–300 g were maintained on Lab-Tek chow and water *ad libitum* and used as both blood and tissue donors. Blood was obtained by inferior vena cava puncture and serum was collected following clot retraction at room temperature. Effective serum calcium concentration was altered by either the addition of calcium chelators (oxalate, citrate, and EDTA), or by the addition of exogenous calcium. In some experiments, exogenous calcium was added to serum pretreated with chelators.

The test colloid used for the in vitro phagocytic studies was the gelatinized "RE test lipid emulsion" prepared as an anhydrous lipid base by blending soya lecithin, glycerol, and ^{131}I-triolein (Mallinckrodt, Nuclear, St. Louis, MO) in a ratio of 1:10:10 by weight, respectively. Prior to use the lipid base was suspended in a gelatin supplemented (0.1%) sterile 5% dextrose and water solution adjusted to pH 7.4, and incubated at 37° with oscillation for 30 min prior to experimentation (2, 3, 19, 20). Previous use of this emulsion for R.E. determinations in animals and humans have documented its active phagocytosis by hepatic Kupffer cells (1, 3, 5).

Hepatic phagocytosis was assayed *in vitro* utilizing a previously described isotopic liver

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slice technique (2, 3, 4, 20). In this technique, 1.0 ml of test serum was combined with 2.0 ml of calcium free Krebs–Ringer phosphate (pH 7.4), 100 USP units of heparin which is essential for active phagocytic uptake (Upjohn, Kalamazoo, MI) and 2000 μg of the gelatinized ^{131}I test lipid emulsion. To this incubation mixture was added a 200–300 mg liver slice prepared with a Stadie–Riggs tissue slicer. The system was incubated under a gas phase of 95% O_2–5% CO_2 at 37° for 30 min with oscillation, and following incubation Kupffer cell phagocytosis of the test colloid was quantified by isotopic means utilizing a Nuclear Chicago deep well gamma counter. *In vitro* hepatic phagocytosis was expressed as the per cent of the initial dose (2000 μg) phagocytized per 100 mg of liver slice (% I.D./100 mg). This technique has been previously used to study the phagocytosis promoting activity of serum from animals and humans (1–6) and an excellent correlation exist between the *in vitro* findings and the *in vivo* functional state of the reticuloendothelial system (1, 2, 20).

Data analysis was accomplished with a digital computer and least squares fit to a straight line as well as a group t test with confidence limits of 95% were employed.

Results. The addition of calcium chelators to normal rat serum manifested a pronounced depressant effect on hepatic phagocytosis. As seen in Fig. 1 a medium devoid of serum shows little ability to stimulate phagocytosis. In contrast, due to the opsonic activity of serum, there is a greater than 20-fold enhancement of phagocytosis when the liver slices are incubated in normal serum. Upon the addition of oxalate, phagocytosis was depressed in proportion to the amount of oxalate added. At the highest concentration of the chelator, (2 μM/ml of serum), phagocytosis was 49.6% ($P < 0.1$) of control with oxalate, 35.8% of control with citrate and 48.3% ($P < 0.01$) of control with EDTA.

To evaluate the potential reversibility of this phagocytic depression, exogenous calcium was added to serum pretreated with oxalate or citrate (Fig. 2). Minimal elevations of the calcium level toward the normal physiologic range resulted in an enhancement of phagocytosis toward normal levels. However, as calcium levels were elevated in excess of physiologic levels there was a significant depressant effect on phagocytosis. At the highest levels of calcium added the observed decrease in phagocytosis was 85.6% ($P < 0.01$) and 76.7% ($P < 0.01$) for oxalate and citrate, respectively.

To separate the effects of increased calcium concentration from the accompanying alterations in ionic strength, calcium chloride and sodium chloride of the same ionic strength were added to separate serum samples (Fig. 3). In contrast to the minimal phagocytic alteration observed in the NaCl

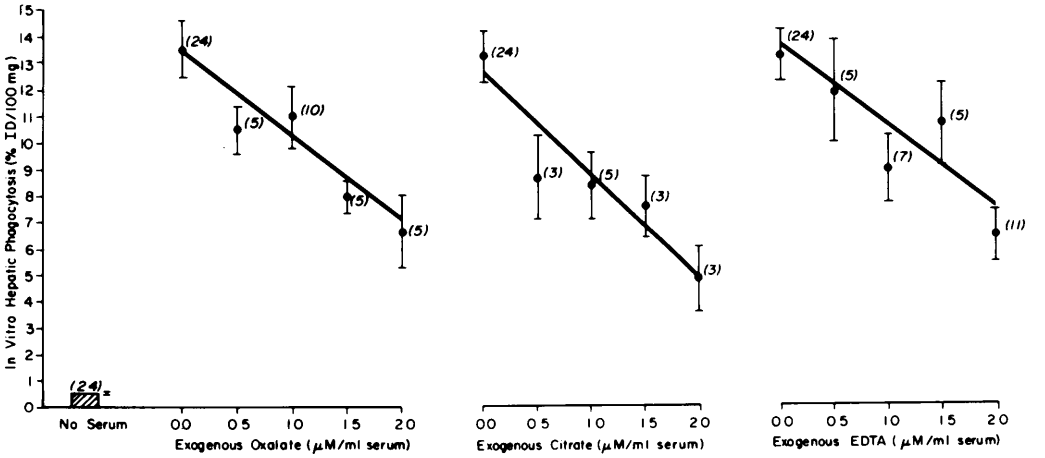

FIG. 1. Hepatic Kupffer cell colloid phagocytosis following addition of exogenous oxalate (left), citrate (middle), and EDTA (right). Data are presented as the mean ± SE, and the lines fitted by the method of least squares. The number of experimental determinations are shown in parenthesis.

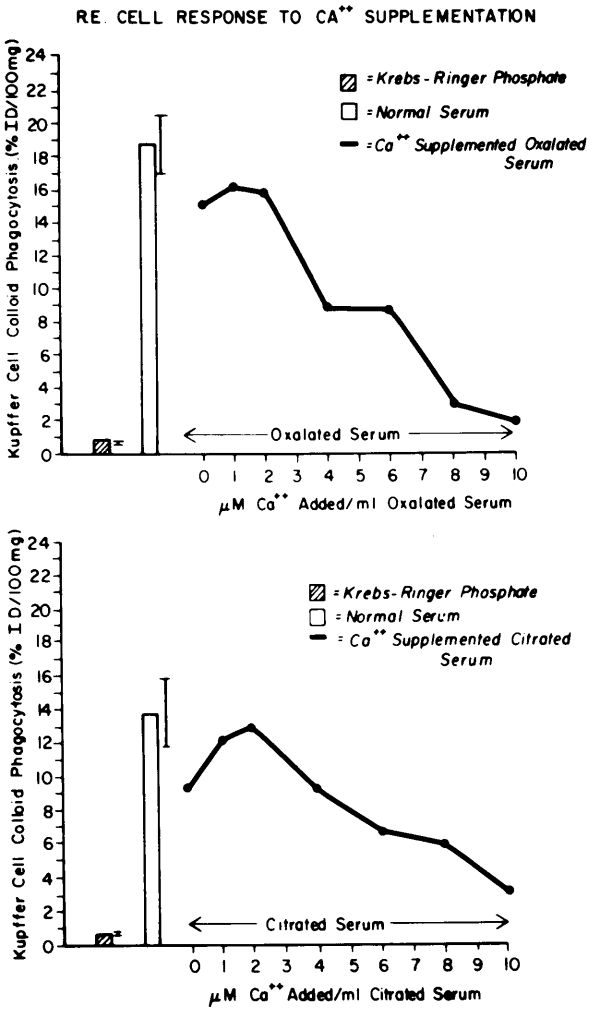

FIG. 2. Hepatic Kupffer cell colloid phagocytosis following addition of exogenous calcium to serum pretreated with oxalate (above) and citrate (below) (1 μM/ml). Experimental points are the mean of three determinations. Controls are given as mean ± SE of the mean.

supplemented samples, there was a significant ($P < 0.05$) decrease in phagocytosis observed in the $CaCl_2$ supplemented samples.

Discussion. Reticuloendothelial phagocytic depression has been documented during the terminal phase of a variety of experimentally induced and spontaneous neoplasms (5, 6, 14, 18, 21). This has been related to a depletion of circulating opsonin levels (6, 18) and/or a functional alteration in the R.E. cell population (21). In both humans and animals, hypercalcemia is associated with a wide variety of tumors (7–12, 15–17). While it was suggested that hypercalcemia was the result of the osteolytic effect of bone metastases, such a concept was challenged by the observations demonstrating frequent hypercalcemia in tumors such as breast carcinomas (11) even in the absence of bone metastases (10).

Since untreated carcinoma of the breast metastasizes readily, a possible mechanism for such spread may be related, in part, to depression of the R.E.S. host anti-tumor defense capacity by increased calcium concentration. Thus, the tumor could be facilitating its own metastatic process via a tumor induced hypercalcemia leading to depression

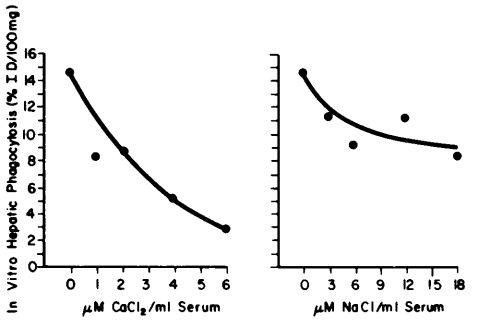

FIG. 3. Hepatic Kupffer cell colloid phagocytosis following experimental manipulation of ionic strength by $CaCl_2$ (left) and NaCl (right). Due to charge differences a NaCl solution three times as concentrated as $CaCl_2$ solution has the same ionic strength.

of the macrophage system. Depression of hepatic phagocytosis in the present studies by elevated calcium levels in the range encountered clinically suggests that this relationship warrants further investigation. While an increase in carbon clearance capacity by the R.E.S. following serial injections of calcium in mice has been reported (22), the time relation between initiation of calcium administration and R.E.S. stimulation (12 hr) leaves question as to whether this is a direct effect of alterations in serum calcium levels.

The phagocytic depressant effects of the calcium chelators as reported in this study are consistent with the observation that citrated blood bank plasma is deficient in opsonic activity (23). In addition, although high levels of calcium depress phagocytosis, it is apparent that calcium is necessary and physiologic levels appear optimal. The fact that human blood bank acid-citrate-dextrose (ACD) blood manifests opsonic activity following Ca^{2+} addition with regard to bacterial phagocytosis (24) supports the presently observed reversibility with respect to hepatic phagocytosis. A qualitatively similar response to calcium concentration has been reported (25) for alveolar macrophages in that some calcium was necessary for phagocytosis but high concentrations induced depression. However, this depressant effect of high calcium was less obvious for a mixed leukocyte population (25).

Whether the calcium effects on phagocytic activity are mediated by a dependency for calcium in the opsonization process or the actual binding and engulfment process remains to be determined. However, the administration of opsonic protein which has been recently isolated and purified will activate the macrophage system and there is an excellent correlation between the *in vitro* phagocytic uptake of the test lipid and the *in vivo* state of the R.E.S. (1, 4, 20). Moreover, the recent demonstration that opsonic protein or so-called recognition factor, as detected by this *in vitro* system, will directly increase tumor resistance *in vivo* (6) supports the concept of an important role for opsonins in the macrophage mediated antitumor defense capacity. Serum calcium may be a critical factor in this opsonin mediated macrophage activation and thus may play a major role in certain nonspecific host defense mechanisms which depend on the process of phagocytosis.

Summary. Serum calcium can markedly influence the phagocytic activity of the hepatic Kupffer cell. Specifically, depletion of calcium by chelators induced impairment of phagocytosis but elevation of serum calcium levels above normal leads to a progressive inhibition of phagocytosis. The findings suggest that alterations of serum calcium may induce significant R.E.S. functional changes which may produce a state of altered host resistance. The relationship of these observations to disturbances of the macrophage system during malignancy, which is known for its ability to alter dramatically the serum calcium concentration, warrants further investigation.

1. Saba, T. M., Arch. Intern. Med. **126,** 1031 (1970).
2. Saba, T. M., Surgery **71,** 675 (1972).
3. Saba, T. M., and DiLuzio, N. R., Amer. J. Physiol. **216,** 197 (1969).
4. Allen, C., Saba, T. M., and Molnar, J., J. Reticuloendothel. Soc. **13,** 410 (1973).
5. Pisano, J. C., Jackson, J. P., DiLuzio, N. R., and Ichinose, H., Cancer Res. **32,** 11 (1972).
6. DiLuzio, N. R., McNamee, R., Olcay, I., Kitahama, A., and Miller, R. H., Proc. Soc. Exp. Biol. Med. **145,** 311 (1974).
7. Hilgard, P., Schmitt, W., Minne, H., and Ziegler, R., Hormones Met. Res. **2,** 255 (1970).
8. Hilgard, P., Hohage, R., Schmitt, W., and Kohle, W., Brit. J. Hematol. **24,** 145 (1973).

9. Tashjian, A. H., Voelkel, E. F., Levine, L., and Goldhaber, P., J. Exp. Med. **136,** 1329 (1972).
10. Plimpton, C. H., and Gellhorn, A., Amer. J. Med. **21,** 750 (1956).
11. Buckle, R. M., McMillan, M., and Mallinson, C., Brit. Med. J. **4,** 724 (1970).
12. Galasko, C. S. B., and Burn, J. I., Brit. Med. J. **3,** 573 (1971).
13. Bradner, W. T., Clarke, D. A., and Stock, C. C., Cancer Res. **18,** 347 (1958).
14. Stern, K. *in* "Reticuloendothelial Structure and Function" (J. Heller, ed.) p. 233 Ronald Press Co., New York, (1960).
15. Ferenczy, A., Okagaki, T., and Richart, R. M., Cancer **27,** 427 (1971).
16. Haskell, C. M., De Vita, V. T., and Canellos, G. P., Cancer **27,** 872 (1971).
17. Gordan, G. S., Fitzpatrick, M. E., and Lubich, W. P., Trans. Ass. Amer. Physiol. **80,** 183 (1967).
18. Saba, T. M., Antikatzides, T. G., and Lorenzen, J. R., J. Reticuloendothel. Soc. **15,** 18a (1974).
19. Saba, T. M., Filkins, J. P., and DiLuzio, N. R., J. Reticuloendothel. Soc. **3,** 397 (1966).
20. Cornell, R. P., and Saba, T. M., Amer. J. Physiol. **225,** 1300 (1973).
21. Old, L. J., Clarke, D. A., Benacerraf, B., and Goldsmith, M., Ann. N. Y. Acad. Sci. **88,** 264 (1960).
22. Bolis, L., Luly, P., and Braun, W., Proc. Soc. Exp. Biol. Med. **136,** 281 (1971).
23. Ollodart, R., and Mansberger, A. R., Amer. J. Surgery **110,** 302 (1965).
24. McCullough, J., Carter, S. J., and Quie, P. G., J. Lab. Clin. Med. **79,** 886 (1972).
25. Stossel, T. P., J. Cell. Biol. **58,** 346 (1973).

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