PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 424–429 (1978)

Increased Lethality After Endotoxin in Old or Leukemic AKR Mice[1, 2] (40069)

SALLIE S. BOGGS[3] AND GRETCHEN N. SCHWARTZ

University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

In the course of studies in which endotoxin (LPS) was given prior to whole body irradiation (WBI) of mice with AKR lymphoma in hopes of obtaining greater damage to the lymphoma (1–3) while promoting earlier regeneration of the normal hematopoietic system (4, 5), it was observed that relatively small doses of LPS killed the older lymphoma bearing AKR mice but not the young controls. Consequently, the present series of studies was carried out to begin to determine the nature of this difference in sensitivity to LPS. Since AKR mice do not develop spontaneous lymphoma until after they reach about 6 months of age the observed sensitivity to LPS could have been due either to the presence of the lymphoma or to an effect of having reached the age at which lymphoma develops. In order to determine which of these factors were involved, comparisons were made of LPS sensitivities of "old" (6–8 months) mice without evidence of lymphoma, "old" mice with spontaneous lymphoma, "young" (3–4 months) mice with transplanted lymphoma and "young" nonlymphoma bearing mice. After these different sensitivities were defined efforts were made to determine whether or not some factors which are known to change with or be associated with responses to LPS might be different in these mice.

Materials and methods. Mice were AKR "young" adult (3–4 months) or "old" (6–8 months) retired breeder or virgin females from either Jackson Laboratories, Bar Harbor, ME, or Charles River Breeding Laboratories, Wilmington, MN, housed 5–10 per cage at about 23–25°C with food and HCl (pH 2.4) water *ad libitum*. During the experiments mice were checked at least once daily and dead animals removed for necropsy. Mice with lymphoma have grossly enlarged spleen, thymus and inguinal lymph nodes at necropsy. The median and ranges of body weights were 27 (23–31) g for 6 month old mice used and 24 (20–28) g for 12-week old mice. Although 6–8 months would be considered to be middle age for many strains, AKR mice have reached 70–90% of their mean life span at 6–8 months and begin to die of spontaneous thymic leukemia, and were therefore defined as "old".

The endotoxin used was *Salmonella typhosa* lipopolysaccharide B 0901 (LPS) from Difco, Detroit, MI. The lyophilized material was diluted to a concentration of 2000 μg/ml in 0.9% NaCl, and stored frozen at −10°C in 25 ml aliquots. The same 2-year old stock solution was used for all of the studies, and was diluted the day before intraperitoneal injection. Aliquots were given as μg LPS per mouse. Death after injection of LPS usually occurred within 1–3 days and data are expressed as 3-day mortality.

The radiation source was a Picker X-ray Unit operating at 200 KV, 15 mA, with 0.25 mm Cu and 1 mm Al filtration. The dose rate of 100 rads/min was calculated from the dose measured by Victoreen dosimetry in air at the center of the plastic mouse container. Dry weights of intestines were obtained as previously described (6).

The transplanted lymphoma was from the 24th and 92nd passage of 10^6 spleen cells collected just prior to death of previously injected mice. Death after each passage occurred within a very tight time span after injection (11 ± 2 days and 7 ± 2 days for the passages used). Endotoxin was injected 5–6 days before the time of expected death.

Reticuloendothelial (RE) function was tested by measuring rates of clearance of carbon from the blood. Colloidal carbon was Gunther Wagner's (Special Ink) #C11/1431A (generously given to us by Dr. Lola Kelly) and prepared according to the

[1] Research supported by a grant from the National Cancer Institute NIH No. 5 R01 CA 16227.

[2] We appreciate the technical assistance of Kenny Patrene and Marilyn Walter.

[3] Leukemia Society of America Scholar.

0037-9727/78/1573-0424$01.00/0

methods described by Halpern *et al.* (7) and Parker and Finney (8). Blood was obtained by puncture of the retro-orbital sinus with a heparinized capillary tube. For platelet counts 50λ of blood was diluted in 100λ of saline, drawn into a segment of Teflon tubing which was sealed at one end, spun at 150 g for 5 min, and platelet rich plasma was diluted in Isoton for electronic counting. Counts were expressed as platelets per mm^3 whole blood. Nucleated cell counts were also obtained by Coulter Counter and differentials were made from 100 Wright stained cells on coverslip blood smears.

Statistics used include, in the case of gut weights, calculation of mean ± 1 SE and, in the case of blood cell counts calculation of geometric mean ± 1 SE. For survival studies, proportions were compared by chi-square test, Wilcoxin tests or Fisher exact confidence calculations where each was appropriate (9). Means and geometric means were compared using the Student's *t* test. Standard errors of percent dead were calculated by $\sqrt{\frac{pxq}{n}}$ where p = percent dead, q = percent alive and n = number of animals tested. A probability of less than $P \leqq 0.05$ was taken to indicate significant differences. LD_{50} data were obtained by probit analysis.

Results. Effect of age. When survival was compared after various doses of LPS given to a total of 212 "old" and apparently nonleukemic mice and 261 "young" mice, it was clear that at the lower doses the "old" mice were much more sensitive to LPS than the "young" mice. Data for "old" virgin females and retired breeder females from Charles River or Jackson Labs were not significantly different and data were pooled. A single injection of LPS was not lethal in young adult mice at doses of 64 μg or less (Fig. 1). In contrast, lethal effects were seen at doses as low as 5 μg in "old" mice. Both curves for young and old mice tended to level off at higher LPS doses, where 256 μg killed only 83% of the "young" mice and 94% of the "old" mice. Probit analysis gave LD50/3 values of 87 ± 50 μg for "young" mice and 28 ± 4 μg for "old" mice.

Effect of lymphoma in "young" mice. If LPS was injected 5–6 days prior to the expected time of death for "young" adult mice with transplanted lymphoma (total of 70 mice) the lethal effects were much greater than in the normal nontransplanted controls. Deaths in the mice with lymphoma were seen with a dose as low as 25 μg LPS and 100% were killed by 100 μg compared to 53% in the normal controls (Fig. 2). Unlike the control curve, the curve for lymphoma mice failed to level off at high LPS doses.

Effect of lymphoma in old mice. Since at 6 months of age some AKR mice begin to show clinical signs of lymphoma and the transplanted lymphoma caused increased sensitiv-

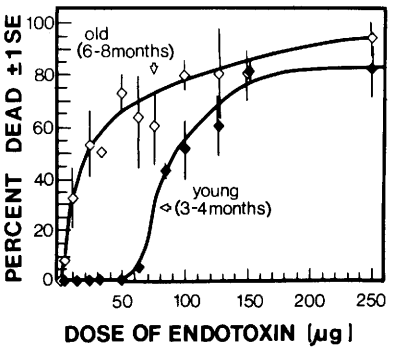

FIG. 1. Percent deaths in young (3–4 months) and old (6–8 months) AKR mice given 1, 5, 10, 16, 25, 32, 50, 64, 75, 100, 128, 150 or 256 μg *Salmonella typhosa* endotoxin. Deaths were cumulative over 3 days after ip injection of the endotoxin into mice. Old mice (open diamonds) were either virgin or retired breeder females. Young mice (closed diamonds) were virgin females. The variations shown were calculated as $\sqrt{pq/n}$. The two curves were significantly different at $P < 0.001$.

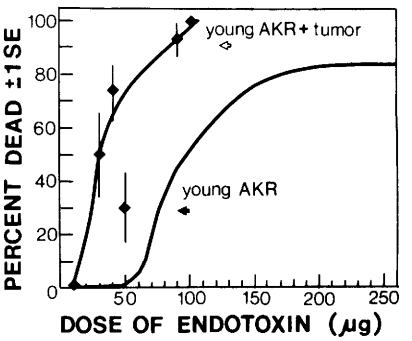

FIG. 2. Percent deaths in young mice without (from Figure 1) and with a growing transplanted AKR lymphoma (closed diamonds) 3 days after various doses of endotoxin. None of the tumor bearing mice died within the 3-day interval if no endotoxin was injected. These curves were different when paired values are compared ($P < 0.001$).

ity to LPS, the status of the "old" mice with regard to lymphoma was determined. Mice with advanced lymphoma were identified by their obviously enlarged lymph nodes and spleen and excluded from our study as they often die just from the trauma of handling. The 74 "old" mice with lymphoma included in the study had either no palpable lymph nodes or spleen, or nodes that were palpable but not grossly enlarged. At death, or at termination of the experiment all the mice were necropsied and classified as either having lymphoma or not. The "old" mice with spontaneous lymphoma showed no evidence of greater sensitivity as compared to "old" mice without lymphoma (Fig. 3). However, the shape of the curves for "old" mice with lymphoma, like that of mice with transplanted lymphoma may have been different from that of mice with no lymphoma in that they failed to level off at high doses of LPS.

Effect of preirradiation. Smith *et al.* (10) have shown that radiation increased sensitivity to LPS injection. This increased sensitivity was greatest 3 days after radiation and was prevented by shielding the gut but not by shielding the marrow from radiation. We tested the combined lethal effects of radiation and endotoxin on "old" and "young" AKR mice (Table I). LPS was injected 3 days after radiation and deaths were recorded for the next 3 days (to 6 days postirradiation). The data provide some evidence that "old" mice were more sensitive to radiation since higher death rates were recorded for "old" than for "young" mice at all radiation doses even when no LPS was given. It is also evident from these data that the combined effects of radiation and LPS are more lethal in "old" than in "young" AKR mice.

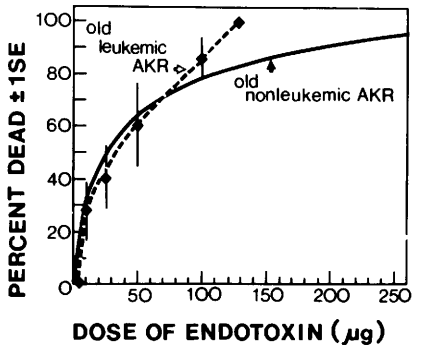

FIG. 3. Percent deaths in old nonleukemic mice (from Fig. 1) and old leukemic mice (closed diamonds) given various doses of endotoxin. These curves were not significantly different ($P \cong 0.5$).

In old mice all doses of radiation resulted in greater sensitivity to endotoxin significant at $P \simeq 0.05$ for 400 rads, $P \simeq 0.005$ for 500 rads, and $P < 0.001$ for both 600 and 700 rads (data in Table I). In young mice only the highest dose (700 rads) resulted in significantly increased sensitivity to endotoxin ($P < 0.001$) (see Table I); 400, 500 or 600 rads failed to do so. No deaths were seen in any of these mice at doses of endotoxin below 32 μg. At 32 μg, 64 μg and 128 μg the combined data for 400 to 600 rads gave 1/40 (3%), 6/30 (20%) and 14/25 (56%) deaths respectively.

Tests for possible causes of different sensitivities. Since previous data suggested that the greater sensitivity to endotoxin seen after radiation might be prevented by gut shielding (10) and since other data have shown that dry weight of the small intestine reflects radiation damage to the mucosal tissue (6), we measured the change in small intestine dry

TABLE I. DEATH OF "YOUNG" AND "OLD" AKR MICE EXPOSED TO RADIATION AND GIVEN *S. typhosa* ENDOTOXIN 3 DAYS LATER.

Endotoxin dose (μg)	Number Dead/Total Tested (%)[a]				
	Old (6–8 months)				Young (3–4 months)
	400 rads	500 rads	600 rads	700 rads	700 rads
0	0/12 (0)	0/13 (0)	4/30 (13)	10/20 (50)	1/15 (7)
1–2	1/6 (17)	2/27 (7)	6/28 (21)	8/23 (35)	4/20 (20)
4–5	2/6 (33)	5/12 (42)	5/13 (38)		0/20 (0)
8	0/6 (0)	2/13 (15)	3/14 (21)		0/10 (0)
16	3/13 (23)	10/14 (71)	9/13 (69)		8/15 (53)
32	9/13 (69)	5/6 (83)	14/14 (100)	14/14 (100)	9/15 (67)
64	12/12 (100)	4/5 (80)	29/29 (100)	13/13 (100)	
128	6/18 (33)	14/14 (100)	29/29 (100)	14/14 (100)	

[a] Nonirradiated control data from these experiments were included in the figures.

weight on days 2, 3 and 5 after 700 rads irradiation. There was no difference in gut weight between the "young" and "old" nonleukemic mice. Weights dropped to 82 and 84% respectively on day 2 and returned to above normal levels on day 5.

Measurement of gut weights of "old" leukemic mice was complicated both by loss of mice due to their greater radiosensitivity and by the presence of enlarged Peyer's patches which become small after irradiation. When these patches were removed before weighing, the pattern of gut weight change began the same (dropping to 82% on day 2) but no mice lived to day 5 and all the weights were lower than for nonleukemic mice.

RE function was tested by determining the rate of removal of colloidal carbon from the blood after iv injection. The half times for carbon clearance were measured in "young" lymphoma bearing mice and non-lymphoma bearing "old" mice. Both were able to remove injected carbon from their blood as rapidly as the "young" controls tested on the same day ($T_{1/2}$ of 34 ± 2 minutes for control and 33 ± 1 for lymphoma and 30 ± 4 for control and 29 ± 1 for "old" groups).

Blood neutrophils and platelets can participate in the removal of injected LPS (11–17) and granulocytes have been said to participate in activation of intravascular coagulation and precipitation of soluble fibrin by endotoxin (18). Blood cell counts in "young" and "old" mice and mice with transplanted lymphoma done 3 days posttransplantation showed significant differences in both the granulocyte and platelet counts. Mice with transplanted lymphoma had higher blood granulocyte counts (5300 ± 610/cubic mm) and lower platelet counts (1.2 ± 0.24 × 10^6/cubic mm) than "young" control mice (1900 ± 200/cubic mm and 3.6 ± 0.24 × 10^6/cubic mm). Similarly "old" mice had somewhat higher granulocyte counts (3000 ± 260/cubic mm) and much lower platelet counts (0.86 ± 0.14 × 10^6/cubic mm) than the "young" controls.

Discussion. The present results indicate that both "old" age and presence of lymphoma can cause greatly increased sensitivity to endotoxin (LPS). The largest differences in sensitivity between "young" and "old" mice were seen at lower LPS doses. At higher LPS doses both groups had some resistant mice. The presence of lymphoma seemed to affect the mice given high as well as low LPS doses. At the lower doses of LPS the presence of spontaneous lymphoma, however, did not increase the sensitivity over that of "old" mice. It seemed possible from this that the "old" mice might have reached a maximum sensitivity. These data may indicate that differences in sensitivity associated with age difference is different than that associated with lymphoma.

There are a number of circumstances which lead to greatly increased sensitivity to LPS. These include, adrenalectomy (19), altered reticuloendothelial function (20–22) or blockade (23), vaccination with BCG (24), serial zymosan injections (25), elevated ambient temperature (26–27), previous irradiation (10), irradiation plus acute graft versus host reaction (17), and immunosuppression (28). When we used one of these, irradiation, it was possible to further increase the sensitivity of the "old" mice. The relative increase in sensitivity due to irradiation, though evident in lower radiation doses in "old" than in "young" mice, was hard to evaluate because the "old" mice proved also to be more sensitive to the radiation.

Many previous studies have shown that "old" mice are more sensitive to radiation than "young" adult mice (29–33) but the strains of mice used in those studies are not "old" or more radiosensitive until they reach about 17–20 months, and in our studies the "old" mice were only 6–8 months old. In terms of life span, however, these ages are more alike. Thus the responses to LPS and radiation of "old" AKR mice may reflect early senescence.

Mechanisms which could be responsible for increased sensitivity to exogenous LPS include increased release of endogenous LPS from the gut (9, 15, 17, 37–45); decreased ability to remove or detoxify LPS (11–17, 20–23, 45–47); increased responsiveness of physiologic mechanism(s) leading to death; and loss of "protective" homeostatic mechanism(s).

There is evidence that the changes in the gut may be involved in these sensitivities. Gut radiosensitivity increases steadily with age from 3 months to 23 months in C57Bl/6J mice (32, 33). Studies of Smith *et al.* (10) showed that radiation-induced increased sen-

sitivity to LPS could be prevented by GI shielding, and other data suggesting changes in mucosal transit time in the gut with aging (34). These, taken together, prompted us to measure postirradiation small intestinal weight changes. In the present studies gut weights decreased to the same degree and increased at the same time and rate in both "old" and "young" mice. Gut weight is a crude measure of mucosal changes after irradiation although it has been useful in other studies (35, 36) in reflecting mucosal damage and regeneration. It is still possible that the vascular or mucosal integrity might be damaged by radiation or injection of LPS and this might lead to more release of endogenous LPS from the gut of "old" or leukemic AKR mice.

In our studies the capacity for RE clearance was studied by determining the $T_{1/2}$ for the removal of colloidal carbon from the blood. The half time for carbon clearance in young lymphoma bearing mice and nonleukemic mice was not significantly different from that of young normal control AKR mice. These studies suggest that RE clearance was normal and that a decreased ability to remove LPS by the RE system is not the mechanism for the observed sensitivity differences.

Blood neutrophil concentration was higher but platelet concentration was lower in "old" mice or young tumor bearing mice as compared to "young" mice. Endotoxin has an affinity for membranes (48) and injected endotoxin rapidly becomes associated with cells of the buffy coat, particularly platelets (11–16). Although the significance of these interactions has not been established, animals which survive LD_{50} doses of endotoxin are able to maintain adequate levels of granulocytes, platelets and coagulation factors, but those that die do not (49). Both granulocytes and platelets appear to have some ability to reduce mortality caused by endotoxin (11–17, 37) but, conversely, both cell types have also been implicated in the development of the pathophysiologic aspects of endotoxemia (18). For example, there is the possibility that elevated granulocyte counts at the time of LPS injection may cause more injury by release of more lysosomal constituents. It is clear that in the progression toward final cardiovascular collapse these cells participate, but it is not clear how the process starts or why it might be worse in "old" or "leukemic" mice.

Summary. Lethality following a single injection of *Salmonella typhosa* endotoxin (LPS) was found to be higher for 6- to 10-month "old" AKR than for "young" adult (3 months old) of the same strain. "Young" AKR mice with transplanted lymphoma were also more sensitive to the lethal effects of endotoxin than the nonlymphomatous "young" mice. Exposure to radiation 3 days prior to endotoxin injection increased sensitivity in both the normal "young" mice and the "old" AKR mice but significant increases were seen at lower radiation doses in the "old" mice. "Old" mice were more sensitive to the radiation than "young" mice. Several possible causes for differences observed were investigated. Reticuloendothelial function as measured by rate of clearance of colloidal carbon from blood was the same for "young" and "old" mice. Previous studies have implicated the gut in the increased sensitivity to endotoxin seen 3 days after radiation. The present studies showed no differences between young and old in degree of small intestine weight loss or rate of recovery after 700 rads X-ray exposure. Although counts of blood granulocytes were somewhat higher and platelets were somewhat lower for the "young" tumor-bearing and "old" mice as compared to "young" controls, it is not obvious how this might affect the differences in LPS sensitivity observed. These data suggest that effects of "old" age, lymphoma, or combined "old" age and radiation may lead to unexpected lethal effects from relatively small doses of endotoxin. The biological reason for this increased sensitivity remains obscure.

1. Jordan, R. T., Rausmussen, A. F., Jr., and Bierman, H. R., Cancer Res. **18,** 943 (1958).
2. Nauts, H. C., Fowler, G. A., and Bogatko, F. H., Acta Med. Scand. **145,** 1 (1953).
3. Watanabe, T., Japan. J. Exp. Med. **36,** 453 (1966).
4. Smith, W. W., Alderman, L. M., and Gillespie, R. E., Amer. J. Physiol. **192,** 549 (1958).
5. Smith, W. G., Brecher, G., Fred, S., and Budd, R. A., Radiation Res. **27,** 710 (1966).
6. Conrad, R. A., Proc. Soc. Exp. Biol. Med. **86,** 664 (1954).
7. Halpern, B. N., Benacerraf, B., and Biozzi, G., Brit. J. Exp. Pathol. **34,** 426 (1950).
8. Parker, H. G., and Finney, C. R., Amer. J. Physiol. **198,** 916 (1960).

9. Landy, M. J., Whitby, J. L., Michael, J. G., Woods, M. W., and Newton, W. L., Proc. Soc. Exp. Biol. Med. **109,** 352 (1962).
10. Smith, W. W., Alderman, L. M., Schneider, C., and Cornfield, J., Proc. Soc. Exp. Biol. Med. **113,** 778 (1963).
11. Braude, A. I., Carey, F. J., and Zalesky, M., J. Clin. Invest. **34,** 858 (1955).
12. Braude, A. I., *in* "Bacterial Endotoxins" (M. Landy and W. Braun, eds.), p. 98, Quinn and Boden Company, Inc., New Jersey (1964).
13. Das, J., Schwartz, A. A., and Folkman, J., Surgery **74,** 235 (1973).
14. Evans, G., Adv. Exp. Med. Biol. **23,** 81 (1971).
15. Palmerio, C., and Fine, J., Arch. Surg. **98,** 679 (1969).
16. Skarnes, R. D., and Rosen, F. S., *in* "Microbial Toxins, V. Bacterial Endotoxins" (S. Kadis, G. Weinbaum, and S. J. Ajl, eds.), p. 151. Academic Press, New York (1971).
17. Walker, R. I., and Sheil, J. M., Exp. Hematol. **4,** 329 (1976).
18. Muller-Berghaus, G., and Eckhardt, T., Blood **45,** 631 (1975).
19. Scott, W. J. M., J. Exp. **39,** 457 (1924).
20. DiLuzio, N. R., and Crafton, C. G., Proc. Soc. Exp. Biol. Med. **132,** 686 (1969).
21. Loose, L. D., Trejo, R., and DiLuzio, N. R., Proc. Soc. Exp. Biol. Med. **137,** 794 (1971).
22. Trejo, R. A., and DiLuzio, N. R., Proc. Soc. Exp. Biol. Med. **136,** 889 (1971).
23. Beeson, P. B., Proc. Soc. Exp. Biol. Med. **64,** 146 (1947).
24. Suter, E., Ullman, G. E., and Hoffman, R. G., Proc. Soc. Exp. Biol. Med. **99,** 167 (1958).
25. Benacerraf, B., Thorbecke, G. J., and Jacoby, D., Proc. Soc. Exp. Biol. Med. **100,** 796 (1959).
26. Jensen, S. B., Mergenhagen, S. E., Fitzgerald, R. J., and Jordan, H. V., Proc. Soc. Exp. Biol. Med. **113,** 710 (1963).
27. Kass, E. H., Atwood, R. P., and Porter, P. J., *in* "Bacterial Endotoxins" (M. Landy and W. Braun, eds.), p. 596, Quinn & Boden Company, Inc., New Jersey (1964).
28. Cuevas, P., Fine, J., and Monaco, A. P., Amer. J. Surg. **127,** 460 (1974).
29. Storer, J. B., Radiation Res. **25,** 435 (1965).
30. Lindop, P. J., and Rotblat, J., Brit. J. Radiation **35,** 23 (1962).
31. Crosfill, M. L., Lindop, P. J., and Rotblat, J., Nature (London) **183,** 1729 (1959).
32. Yuhas, J. M., and Storer, J. B., Radiation Res. **32,** 596 (1967).
33. Yuhas, J. M., Huang, D., and Storer, J. B., Radiation Res. **38,** 501 (1969).
34. Lesher, S., Fry, R. J. M., and Kohn, H. I., Exp. Cell Res. **24,** 334 (1961).
35. Fred, S. S., Wilson, S. M., and Smith, W. W., "Excerpta Medica Monographs on Nuclear Medicine and Biology No. 1" report of a Symposium, held at Richland, Washington, p. 413 (1966).
36. Torok, B. J., and Boggs, S. S., Amer. J. Physiol. **228,** 1280 (1975).
37. Cuevas, P., and Fine, J., J. Reticuloendothel. Soc. **11,** 535 (1972).
38. Gans, H., and Matsumoto, K., Surg. Gynecol. Obstet. **139,** 395 (1974).
39. Jensen, S. B., Mergenhagen, S. E., Fitzgerald, R. J., and Jordan, H. V., Proc. Soc. Exp. Biol. Med. **113,** 710 (1963).
40. Schaedler, R. W., and Dubos, R., *in* "Bacterial Endotoxins" (M. Landy and W. Braun, eds.), p. 390, Quinn and Boden Company, Inc., New Jersey (1964).
41. Walford, R. L., J. Immunol. **117,** 352 (1976).
42. Yoshida, M., Hirata, M., Homma, J. Y., Abe, C., Tajima, Y., and Maejima, K., Japan. J. Exp. Med. **46,** 297 (1976).
43. Fred, R. K., Dobson, E. L., Kelly, L. S., and Shore, M. L., J. Reticuloendothel. Soc. **7,** 453 (1970).
44. Walker, R. I., Moon, R. J., Alm, P. F., and Ledney, G. D., Experientia **32,** 1527 (1976).
45. Fust, G., and Foris, G., Med. Microbiol. Immunol. **159,** 141 (1974).
46. Morrison, D. C., and Kline, L. F., J. Immunol. **118,** 362 (1977).
47. O'Flaherty, J. T., Craddock, P. R., and Jacob, H. S., Proc. Soc. Exp. Biol. Med. **154,** 206 (1977).
48. Hildebrand, G. J., and Seys, Y., Nature (London) **206,** 406 (1965).
49. Altura, B. M., and Hershey, S. G., Circ. Shock **1,** 113 (1974).

Received October 7, 1977. P.S.E.B.M. 1978, Vol. 157.