described which can be correlated with the precipitin method for antiurease.

1. Kirk, J. S., Sumner, J. B., *J. Biol. Chem.*, 1931, v94, 21; ———, *J. Immunol.*, 1934, v26, 495.

2. Marrack, J. R., *The Enzymes*, ed. J. B. Sumner & K. Myrback, 1951, v1, Academic Press, N. Y., 436.

3. Cinader, B., *Ann. Rev. Microbiol.*, 1957, v11, 371.

4. Marucci, A. A., Mayer, M., *Arch. Biochem.*, 1955, v54, 330.

5. Neter, E., *Bact. Rev.*, 1956, v20, 166.

6. Cole, L. R., Farrell, V. R., *J. Exp. Med.*, 1955, v102, 631.

McKenna, J. M., PROC. SOC. EXP. BIOL. AND MED., 1957, v95, 591.

7. Ingraham, J. S., *ibid.*, 1958, v99, 452.

8. Dounce, A. L., *J. Biol. Chem.*, 1941, v140, 307.

9. Sumner, J. B., *ibid.*, 1926, v69, 435.

10. Dang, H. C., Visek, W. J., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 164.

11. Csizmas, L., *ibid.*, 1960, v103, 157.

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## Accelerated Rejection of Male Skin Isografts by Female C57BL Mice Infected with *Bacillus Calmette-Guérin* (B.C.G.).* (27103)

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Several methods to alter the homograft reaction in a non-specific‡ fashion have been described. Depression of immunologic reactivity as measured by increased survival of grafted tissue has been accomplished with x-rays, cortisone and certain drugs, the degree of success largely depending on experimental conditions(1). On the other hand, little attention has been paid to the possibility of non-specifically accelerating the homograft mechanism. Increased resistance to transplantable homologous and isologous tumors in mice has been demonstrated following zymosan and B.C.G. infection(2-5). The effect was attributed to a host mediated immune response, rather than a direct action on the tumors. This assumption was made in view of the fact that both agents increase immunological reactivity as measured by other means: prior administration of B.C.G. or zymosan enhances the phagocytic activity of the reticuloendothelial system (RES), increases the host's capacity to form antibody, and heightens resistance to infectious challenge(6-12).

To substantiate the assumption that increased host resistance to tumor growth following B.C.G. and zymosan is mediated by a heightened capacity to reject antigenic grafted tissue, it was felt important to demonstrate comparable effects using transplantation of normal tissue.

The present report deals with the effect of B.C.G. on rejection time of male skin isografts by C57BL female recipients.

*Materials and methods. Mice.* Adult male and female C57BL/6 mice were obtained from Bio-Research Consultants, Inc., Cambridge, Mass. and from a colony of C57BL mice originally obtained from Dr. Daniel S. Martin, Univ. of Miami, and maintained in this laboratory on a strictly inbred basis for the last 2 years. Female mice were grafted exclusively with skin from male mice of the same colony, although reciprocal grafts proved the mice from both sources to be isologous.

*B.C.G.* The Phipps strain of *Mycobacterium tuberculosis* var. *Bacillus Calmette-Guérin* (B.C.G.) was grown in Dubos fluid medium. Pooled 10- to 14-day cultures were harvested by centrifugation, weighed and di-

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‡ "Non-specific" refers to methods that alter rejection time of transplanted antigenic tissue without prior introduction of specific cellular or non-cellular antigens, sensitized cells or specific antibody.

luted to 5 mg/ml wet weight. One mg (0.2 ml) of the B.C.G. suspension was injected into the tail veins (i.v.) of mice receiving the live organisms by this route. A suspension containing 20 mg/ml was prepared for intracutaneous injections. A small area on the back, between the left inguinal lymph node and the dorsal midline was shaved and 1 mg of B.C.G. (0.05 ml) was injected intracutaneously (i.c.). Skin grafts were subsequently placed approximately 1 cm from site of injection, on the lateral chest wall.

*Skin grafting.* Transplantation was performed according to the technic of Billingham and Medawar(13). Pieces of skin 1 × 2 cm in size, in the non-active stage of hair growth, were grafted on the left lateral chest wall of control non-infected mice, and 2 weeks after i.v. or i.c. administration of B.C.G. The plaster dressings were removed on day 11 and the grafts were inspected first daily, later every other day. Only 2 experimental animals had to be excluded from the evaluation due to slight damage of the graft on the day of removal of the dressing. The day when only atrophic scar tissue remained was scored as the rejection time, but the time interval from initiation until end of the rejection process was also recorded (Fig. 1).

*Results.* Rejection of male skin isografts by female recipients occurred in about 95% of the untreated C57BL mice used in the present experiment. The process of graft rejection began with loss of hair, increasing pallor, scab formation and gradual shrinking of the graft until only a tiny patch of scar tissue remained. Hemorrhage and ulceration of the graft were not frequently seen in these control animals. It took at least one, but sometimes several weeks from the first macroscopic evidence of damage until complete rejection of the graft, and this endpoint occurred from 6 to 12 weeks after transplantation. Precise determination of the survival endpoint was at times difficult.

Significant reduction of the rejection time was observed in female C57BL recipients receiving male isografts 2 weeks following administration of B.C.G., intravenously or intracutaneously (Fig. 1). The rejection endpoint occurred, as an average, 2 weeks earlier

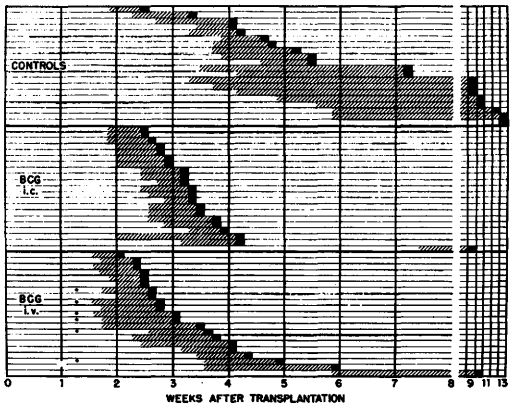


FIG. 1. Pattern of rejection of male skin isografts on B.C.G. infected female C57BL mice. Each bar represents one experimental animal. Shaded area depicts interval between initiation of rejection to complete graft destruction (black square in figure). One control animal (×) did not reject the male isograft during observation period of 6 mo.

than in controls. In addition, the speed of the rejection process, from the first evidence of damage until graft destruction, was also accelerated. B.C.G. treated animals showed a more fulminating type of rejection, with hemorrhage and ulceration of the isografts, quite reminiscent of the appearance of skin homografts during rejection.

The standard dose of 1 mg B.C.G. was chosen for i.v. administration since previous experiments had shown this amount to be effective in causing a marked and prolonged increase of resistance to a variety of challenges(3,10,14). As a rule, morbidity or mortality is not observed following this dose in most strains of mice. However the subline of C57BL mice bred in our laboratory did not tolerate intravenous B.C.G. injection as evidenced by marked weight loss and occasional death. Such debilitated mice did not show accelerated rejection of male isografts, and were not included in evaluating the results. Intracutaneous administration of B.C.G., however, had no observable ill effect on the physical condition of these mice, and accelerated rejection of male isografts was as consistent as in the other subline.

It is known that draining lymph nodes play a crucial part in destruction of homografts (15,16). Intravenous administration of B.C.G. primarily affects the reticuloendothel-

ial cells in liver and spleen and does not produce important histological changes in the lymph nodes. Therefore it was thought that if B.C.G. were applied in such a manner as to predominantly affect regional lymph nodes, accelerated graft rejection might also occur. Two weeks after injection of 1 mg of B.C.G. into the dorsal skin of mice, significant enlargement of the regional lymph nodes (inguinal, brachial and axillary) is observed. Microscopically these nodes reveal macrophage hyperplasia and granuloma formation. Male skin grafted onto females at a distance from the site of such an intracutaneous injection, but still within the same area of lymphatic drainage, was rejected at an accelerated rate, similar to the rejection pattern following intravenous B.C.G. administration. It is to be emphasized that initial healing-in of male isografts on female recipients is uninfluenced by intravenous or intracutaneous B.C.G. infection. Control groups of female isografts on female recipients survived undisturbed following B.C.G. by either route and were indistinguishable from grafts on non-infected controls.

A number of intravenously injected mice (* in the figure) received an additional intracutaneous dose of B.C.G. one week after an i.v. injection. The regional lymph nodes again showed the expected enlargement. If male isografts were then applied one week later, 2 weeks after the original i.v. injection, rejection of the grafts was accelerated to the same extent as following i.v. injection alone.

Five animals were splenectomized one week following intravenous injection of B.C.G.; whereas splenectomy did not alter rejection time of male isografts in a group of 9 uninfected female controls, the accelerated rejection of such grafts by intravenously infected recipients was abolished by splenectomy. These experiments are currently being repeated with larger numbers of animals.

Although the relationship between time of B.C.G. administration and subsequent grafting was not fully investigated, it was observed that acceleration of rejection was less evident if females received male skin grafts one week following B.C.G. administration.

*Discussion.* B.C.G. infection in mice has been shown to improve the host response to a variety of antigenic and infectious challenges. These include heightened resistance to growth of transplanted tumors as well as to bacterial and viral infection. Following i.v. injection of B.C.G., enhanced phagocytic activity of the RES as well as increased capacity to produce antibodies and opsonins has been observed(6,8). Increased numbers of phagocytic cells in liver and spleen, partly through transformation of endothelial cells, but also due to a proliferative stimulus exerted on existing reticuloendothelial cells are evident(17). Liver and spleen of these animals are markedly enlarged, mainly due to granuloma formation of phagocytic elements in both organs as well as to large numbers of pyroninophylic cells in the spleen.

The observation of increased resistance to transplantable antigenic tumors following B.C.G. infection(3-5) made it desirable to demonstrate similar accelerated rejection in transplantation of homologous non-malignant tissue. In unpublished experiments we have been unable to observe any macroscopic difference in rejection time of homografted skin between B.C.G. treated recipients and controls; neither were microscopic differences evident. Normal survival times of such grafts are short (10 days), leaving little room for detection of minor curtailment in survival of the grafts. A slower type of rejection seemed therefore a more desirable method of assay.

Since the observation by Eichwald and Silmser(18), that females of certain mouse strains consistently reject male isografts, it has been adequately demonstrated that such rejection is in response to a histocompatibility antigen associated with the Y chromosome and that the mechanism of rejection, although of a more chronic nature, is essentially similar to that of homografts(19). The ability of females to reject male tissue is genetically determined(20,21). Consistent rejection (95%) of male isografts by C57BL females, added to their high immunological reactivity in general, made this strain an ideal tool to reveal subtle differences in rejection time.

Accelerated rejection of male skin isografts by B.C.G. infected female C57BL mice was

in fact demonstrated in the present study. Although endocrine or nutritional factors cannot completely be excluded, it appears most likely that alterations in the immune response following B.C.G. infection in some manner influence the homograft mechanism. Rejection of antigenic grafted tissue may conveniently be divided into a number of phases (16,22). This immunological process is believed to begin with the passage of antigen to the regional lymph nodes and other lymphatic tissue such as the spleen. There it is presumed to be recognized following phagocytosis or by other means, and subsequently production of immunologically active lymphoid cells occurs. After passage of these cells to the graft site, interaction with the transplanted tissue takes place. The role of humoral antibody remains controversial in this process although it is known to be important in rejection of certain tumor grafts(23,24). B.C.G. infection results in a greater number of functionally altered phagocytic cells and it exerts a proliferative stimulus on other cells of the reticuloendothelial system; it is therefore conceivable that the phase of antigen recognition and degree of immunological response may be primarily influenced. Increased speed of sensitization and more vigorous production of immunologically competent cells might be essential factors in faster graft rejection mediated by B.C.G.

Similar reasoning can probably be applied to the mechanism whereby tumor inhibition is noted following B.C.G. infection in mice. Certain differences in the behavior of the two systems, however, can be noted. Whereas male skin isografts were rejected at an accelerated rate following intracutaneous injection of B.C.G., growth of Sarcoma 180, transplanted subcutaneously, was not influenced under similar circumstances. In contrast, intravenous inoculation of B.C.G. does lead to more prompt rejection of both tumor and skin grafts. Another difference in behavior of the two systems was observed following splenectomy of animals intravenously infected with B.C.G. Splenectomy appeared to abolish the accelerated rejection of male skin by B.C.G. infected females but did not alter the increased resistance to transplanted Sarcoma 180 following B.C.G. infection. The ability of tumors to override the heightened regional immune mechanisms induced by an intracutaneous injection of B.C.G., and the possible difference in importance of the spleen in rejection of tumor and skin grafts, may account for the dissimilarities in behavior between the two tissues.

*Summary.* Accelerated rejection of male skin isografts by B.C.G. infected female C57 BL mice was demonstrated. Skin was grafted 2 weeks following intravenous or intracutaneous injection of 1 mg B.C.G. This accelerated rejection was compared to previously reported inhibition of tumor growth in B.C.G. infected mice. Whereas it is not known at what phase of the rejection mechanism B.C.G. exerts its influence, it now appears likely that increased resistance to the grafted tissue is in both instances mediated by a heightened immune response.

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1. Brent, L., *Progress in Allergy,* 1958, v5, 271.
2. Bradner, W. T., Clarke, D. A., Stock, C. C., *Cancer Res.,* 1959, v19, 673.
3. Old, L. J., Clarke, D. A., Benacerraf, B., *Nature,* 1959, v184, 291.
4. Biozzi, G., Stiffel, C., Halpern, B. N., Mouton, D., *Compt. rend. soc. biol.,* 1959, v153, 987.
5. Old, L. J., Benacerraf, B., Clarke, D. A., Carswell, E., Stockert, E., *Cancer Res.,* 1961, v21, 1281.
6. Biozzi, G., Benacerraf, B., Grumbach, F., Halpern, B. N., Levaditi, J., Rist, N., *Ann. Inst. Pasteur,* 1954, v87, 291.
7. Benacerraf, B., Sebestyen, M. M., *Fed. Proc.,* 1957, v16, 860.
8. Halpern, B. N., Biozzi, G., Stiffel, C., Mouton, D., *Compt. rend. soc. biol.,* 1958, v152, 758.
9. Cutler, J. L., *Fed. Proc.,* 1959, v18, 33.
10. Dubos, R. J., Schaedler, R., *J. Exp. Med.,* 1957, v106, 703.
11. Rowley, D., *Lancet,* 1955, v1, 232.
12. Old, L. J., Clarke, D. A., Stockert, E., Porter, C., Orenski, S. W., *Fed. Proc.,* 1961, v20, 265.
13. Billingham, R. E., Medawar, P. B., *J. Exp. Biol.,* 1951, v28, 358.
14. Balner, H., Old, L. J., Clarke, D. A., *Radiation Research,* 1961, v15, 836.
15. Mitchison, N. A., *Nature,* 1953, v171, 267.
16. Medawar, P. B., *Cellular and Humoral Aspects of Hypersensitivity States,* 1959, ed. H. S. Lawrence, N. Y., Hoeber.

17. Thorbecke, G. J., Old, L. J., Benacerraf, B., Clarke, D. A., *J. Histochem. and Cytochem.*, 1961, v9, 392.

18. Eichwald, E. J., Silmser, C. R., *Transpl. Bull.*, 1955, v2, 148.

19. Krohn, P. L., *ibid.*, 1958, v5, 126.

20. Hauschka, T. S., Grinell, S. T., Meagher, M., Amos, D. B., *Genetics and Cancer*, 1959, ed. R. W. Cumley *et al.*, Univ. of Texas Press, Austin.

21. Zaalberg, O. B., *Biological Problems of Grafting*, 1959, v12, 306, In Liége Univ.

22. Snell, G. D., Winn, H. J., Kandutsch, A. A., *J. Immunol.*, 1961, v87, 1.

23. Gorer, P. A., Amos, D. B., *Cancer Res.*, 1956, v16, 338.

24. Stetson, C. A., Jensen, E., *Ann. N. Y. Acad. Sci.*, 1960, v87, 249.

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## Experimental Studies of Factors Influencing Hepatic Metastases: XI. Effect of Hepatic Trauma in Hypophysectomized Animals.* (27104)

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We have reported(1) that hepatic trauma greatly augmented the take and growth of experimentally induced hepatic metastases. The same results were obtained in adrenalectomized animals(2), indicating to us that adrenocortical "stress" was probably not responsible for this phenomenon. Recently we noted that the incidence of such tumors was markedly reduced in hypophysectomized animals and that suppression of metastases appeared independent of the general somatic response to hypophysectomy or to deficiency of ACTH(3). This provoked the question as to whether or not the augmentory tumor response to trauma was eliminated in pituitary ablated animals or whether or not the suppressive tumor effect of hypophysectomy could be overcome by liver damage. The following presents the effect of hepatic trauma upon artificially induced hepatic metastases in hypophysectomized animals.

*Materials and methods.* Adult female normal and hypophysectomized Sprague-Dawley rats‡ weighing 175-200 g were used in all experiments. They were maintained in individual cages and permitted water *ad libitum*. Hypophysectomized animals were received in the laboratory 2 days after operation. Upon arrival both hypophysectomized and control animals were placed on a diet of one-half ground Chow§ and one-half dog food‖ with a daily supplement of fresh orange and raw carrot. Following 8 days of *ad libitum* feeding, paired-feeding of normal controls to non-traumatized hypophysectomized animals was begun and maintained until sacrifice. The latter continued to eat *ad libitum*. After a week of such feeding, groups of normal control animals, hypophysectomized control rats and pituitary ablated animals to be subjected to hepatic trauma were injected intraportally with Walker carcinoma cells. This tumor has been propagated in our laboratory for 4 years. The method of tumor cell preparation and inoculation has been described(4).

All animals in these experiments were lightly anesthetized with ether, subjected to celiotomy, and injected intraportally *via* a large mesenteric vein with ½ ml of a saline-plasma solution containing 5,000 tumor cells. Experiments were devised so that control and experimental animals received injections of the same tumor cell preparation. Hepatic trauma, as previously reported(1), was produced by massage of the liver either 5 minutes, 2, 7, or 14 days following injection of tumor cells, and animals were sacrificed 14 days following the manipulation. Careful gross examination of livers for tumor was

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‡ Hormone Assay Laboratories, Inc., Chicago, Ill.

§ Purina.

‖ Ken-L-Ration.