INFLUENCE OF SPLENECTOMY ON FIRST SET REJECTION REACTIONS OF C57BL/6 FEMALES TO MALE SKIN ISOGRAFTS*

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Descriptions of unique and definitive roles for the spleen in immunologic responses have generally proven to be ephemeral. The vulnerability of any hypotheses claiming a special role for this organ relates to the fact that animals are usually able to survive reasonably well after splenectomy. Therefore, a unique role for the spleen analogous to that of the thymus or the bursa of Fabricius seems exceedingly unlikely. However, as factors responsible for regulation of the immune response have been more thoroughly studied, renewed possibilities for special functions of the spleen have emerged.

Three major areas of investigation have indicated that the spleen plays a unique role in immunologic responses: (*a*) Humoral antibody responses to intravenously inoculated antigens, especially particulate. The classic work of Rowley on rabbit antibody responses to heterologous erythrocytes defined this area (1, 2); (*b*) protection against certain protozoal infestations which are more devastating and/or disseminated in the absence of the spleen (3); and (*c*) production of enhancing antibodies and/or factors which promote the growth or survival of tissue allografts and autochthonous tumors, a role for the spleen initially postulated by Prehn (4) and studied extensively by Gershon and Carter (5), and Ferrer (6). The major thrust of these lines of investigation has been to delineate prominent roles for the spleen in antibody production to intravenously inoculated antigens and as a site for the removal by opsonization and phagocytosis of microorganisms by the reticuloendothelial system.

More recently, attention has been directed to the duality of the immunologic response in which sometimes antagonistic, sometimes synergistic activities of cell-mediated and antibody-mediated immune effector mechanisms co-exist. In elegant studies of the interaction of these two effector modalities, Lagrange et al. and Mackaness et al. (7, 8) have shown that the spleen can play a pivotal role in determining the overall manifestation of immunologic responsiveness. In this laboratory, evidence has recently been produced which suggests that when lymphoid cells bearing allogeneic antigens are placed into the anterior chamber of the rat eye, an immunologically privileged site, the speed with which alloimmunity as expressed by skin graft rejection develops depends upon the presence or absence of an intact spleen (9, 10). Splenectomy before anterior chamber inoculation of allogeneic cells permits intense immunity on the part of the host, reflected by shortened skin graft survival times. Stimulated by these observa-

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tions, experiments have been conducted to investigate whether the spleen plays a role in determining the tempo of first set allograft rejection. In the experiments to be described, the response of C57BL/6 female mice to isogeneic male skin grafts has been analyzed with regard to the effect of prior splenectomy. The results indicate that in this system of weak antigenic disparity, the spleen does play an important role in determining the speed with which grafts are rejected apparently by providing a suppressing influence upon the mediators of graft rejection.

Materials and Methods

Animals. C57BL/6 male and female mice used in these experiments were obtained from The Jackson Laboratory, Bar Harbor, Maine. All animals were adults, between 2 and 4 mo of age.

Lymphoid Cell Suspensions. Lymphoid cell suspensions were prepared from lymph nodes and spleens by the method previously described (11). Cells for inoculation intracutaneously, intraperitoneally, or into the hind foot pads were adjusted to the appropriate viable cell concentration in Hanks' balanced salt solution.

Skin Grafts. Skin grafts obtained from body wall were placed orthotopically by the method of Billingham and Silvers (12). Dressings were removed at 7 days and the degree of epithelial survival scored daily until rejected. Complete epidermal necrosis was regarded as the survival end point. Median survival times (MST),[1] standard deviation and error of the mean, and 95% confidence limits were derived from the method described by Litchfield (13).

Splenectomies. Splenectomies were carried out through a left lateral flank incision under ether anesthesia. Sham operated controls had their spleens exteriorized through the abdominal wall wound, then replaced into the peritoneal cavity.

Gamma Irradiation. Gamma irradiation of mice was accomplished in a Gamma-Cell 40 cesium-137 source at a rate of 105 rads/min.

Popliteal Lymph Node Assays. Popliteal lymph node assays were conducted as described by Ford et al. (14). Briefly, 5×10^6 donor lymphoid cells were inoculated into the hind foot pad of sex-, age-, and weight-matched recipients. 7 days later the draining popliteal lymph nodes were extirpated and weighed.

Experiments and Results

Effect of Splenectomy on First Set Skin Graft Rejection Time. In the first series of experiments, C57BL/6 female mice were splenectomized or sham operated. 30 days later, they were challenged with orthotopic grafts from syngeneic male donors. The results of these experiments are listed in Table I. MST of male skin on splenectomized females was 13 days. By contrast, the MST on sham operated animals was 16.5 days. The difference between these two groups is statistically significant. More impressively, male grafts placed on females that had been splenectomized only 7 days previously displayed even shorter survival times, the MST of these grafts being reduced to 9.0 days. This very brisk rejection reaction was comparable to that of specifically hypersensitized females (15) and was as rapid as if a major *H* locus incompatibility prevailed.

Influence of Replacement of Spleen Cells in Splenectomized Hosts. In the following series of experiments, evidence was sought bearing on the possibility that splenectomy removes a population of cells with the capability of suppressing first set allograft responses. In these studies, panels of C57BL/6 females were splenectomized 7 days before receiving grafts of male skin. 2 days thereaf-

[1] *Abbreviations used in this paper:* GVHR, graft-vs.-host reactivity; F-anti-M, female-anti-male; LNC, lymph node cells; MST, mean survival time; Splx, splenectomized.

TABLE I
Effect of Splenectomy on First Set Skin Graft Survival

Experimental animals	No.	Median survival time (days ± SD)*
Sham operated (control)	39	16.5 ± 1.3 (15.1–18.0)
Splenectomized 30 days before grafting	22	13.0 ± 1.1 (11.7–14.6)
Splenectomized 7 days before grafting	30	9.0 ± 1.6 (7.7–10.6)

Adult C57BL/6 females were grafted orthotopically with isografts of male skin.
* Numbers in parentheses are 95% confidence limits.

TABLE II
Adoptive Restoration in Splenectomized C57BL/6 Females of First Set Reactivity to Male Skin Grafts

Cell transfer donors	Cell type transferred	No. of splenectomized recipients	Median survival time (days ± S.D.)*
Normal C57BL/6 female	Spleen	15	17.0 ± 1.5 (13.7–21.1)
Normal C57BL/6 female	Lymph node	4	9.0 (8–11)‡
C57BL/6 females grafted with male skin 10 days before sacrifice	Spleen	13	11.5 ± 1.2 (10.8–12.2)
C57BL/6 females grafted with male skin 5 days before sacrifice	Spleen	16	18.0 ± 1.5 (14.6–22.2)
None (control)	–	30	9.0 ± 1.6 (7.7–10.6)

* Numbers in parentheses are 95% confidence limits.
‡ Range of survival times.

ter, these animals received intraperitoneally suspensions of spleen cells and lymph node cells from normal female donors and from eusplenic female donors that had received male skin grafts 10 days before sacrifice. Each host received one donor equivalent of lymph nodes or spleen cells. The results of these experiments, which are displayed in Table II, reveal that spleen cells from normal females were able to reconstitute the first set allograft response in splenectomized recipients, restoring the MST of male skin grafts to 17.0 days. By contrast, suspensions of neither normal lymph node cells nor lymph node cells and spleen cells from females grafted 10 days previously with male skin had comparable effects on the survival of test male skin grafts in splenectomized females. We interpret this to mean that there is a resident population of cells in the spleen before antigenic challenge which is capable of suppressing the development of the allograft immune response to the H-Y antigen. The results further suggest that these cells are not present in lymph nodes of normal females and are depleted from lymph nodes and spleens of females grafted with male skin 10 days beforehand.

A subsequent experiment was carried out identical to the previous one except for the fact that the donors of spleen cells were females who had received male skin grafts 5 days before sacrifice. The results which are also displayed in Table II indicate that spleen cells at this shorter interval after skin allografting are still capable of suppressing the development of immunity and the MST of male grafts on splenectomized recipients is restored to 18 days.

TABLE III

Systemic GVH Reactivity of Lymphoid Cells from Splenectomized C57BL/6 Female-Anti-Male Animals

Recipient	No.*	Donor	Change in body weight	Spleen wts (mg)/ body wts (g) (±2 SEM)
			%	
Normal male	(8)	F-anti-M LNC	+18	3.02 ± 0.38
300 rad male sham splenectomized	(11)	F-anti-M LNC	+19	2.59 ± 0.16
Normal male (control)	(10)	None	–	3.20 ± 0.50
300 rad male splenectomized	(15)	F-anti-M LNC	+26	–
300 rad male	(5)	None	+10	–

Adult C57BL/6 males received intracutaneously 50 × 10^6 lymph node cells (LNC) from females previously splenectomized and highly sensitized to H-Y antigen.

* Number of recipient animals.

TABLE IV

Local GVH Reactivity of Lymphoid Cells from Splenectomized C57BL/6 Female-Anti-Male Animals

Recipient	No.*	Donor	Popliteal lymph node weight (mean ± 2 SEM)
Normal male	(34)	None (control)	1.32 ± 0.16
Normal male	(47)	Normal female	1.61 ± 0.14
Normal male	(38)	F-anti-M Sham Splx	2.77 ± 0.34
Normal male	(30)	F-anti-M Splx	1.26 ± 0.14

Recipients received 5 × 10^6 donor cells into the hind foot pad. Popliteal lymph nodes were removed and weighed 7 days later.

* Number of popliteal lymph nodes weighed.

Graft-Vs.-Host Reactivity of Cells from Specifically Sensitized Normal and Splenectomized Females. Because of the vigor with which splenectomized females rejected first set skin allografts from male donors, we decided to evaluate their lymphoid cells for graft-vs.-host reactivity (GVHR). Accordingly, females that had been splenectomized or sham operated and that had rejected male skin grafts, were boosted 30 days later with a total of 30 × 10^6 male spleen cells in three intracutaneous injections. 7 days later their lymph nodes were removed and single cell suspensions assayed for GVHR in two ways: (*a*) Aliquots totaling 50 million cells were inoculated in five injection sites intracutaneously in normal males, and in sham operated males and splenectomized males exposed to 300 rad on the day of inoculation. Normal unirradiated males served as control. (*b*) Panels of normal C57BL/6 males were inoculated into the hind foot pad with 5 million lymph node cells from sham operated or splenectomized female donors that had rejected male skin and were boosted 7 days before. The results of these experiments are displayed in Tables III and IV. After intracutaneous inoculations of donor cells, none of the male animals were observed to exhibit generalized skin eruptions or other clinical manifestations generally associated with GVH disease. As Table III indicates, all panels of animals

gained weight during the course of the experiment. Splenic weights from eusplenic animals failed to document hypertrophy, as another measure of GVHR. However, in local assays of GVHR (Table IV), we were surprised to discover that cells harvested from specifically sensitized females who had been sham operated evoked significant hypertrophy in draining popliteal lymph nodes while cells from splenectomized and specifically sensitized females failed to elicit significant popliteal node enlargement. We conclude from these experiments that C57BL/6 females with intact spleens respond to allografts of male skin by generating within their lymphoid tissues increased numbers of cells capable of mediating GVHR. Alternatively, the lymphoid mass of females without a spleen appears to be enriched for cells capable of mediating acute skin graft rejection, but peculiarly devoid of GVH potential.

Discussion

Claims for a unique role of the spleen in immunologic responses are not new. It has been determined that for particulate antigens administered intravenously in virgin animals the spleen is the primary site of antibody synthesis. As a consequence of this, the spleen plays a pivotal role in protection against hematogenous spread of pathogenic organisms under selected experimental conditions. In addition, the spleen was purported to play a dominant role in the production of enhancing antibodies as described by Prehn in 1959 (4). A variety of experiments carried out subsequently have merely revealed that while the spleen may be an important source for enhancing antibodies with respect to tumor and normal tissue allografts, it is clearly not the only source. Enhancement can be achieved in the absence of an intact spleen (16).

More recently, the spleen has been regarded as a site of lymphocyte differentiation and it has been suggested that the maturation of B lymphocytes as evidenced by the appearance of IgD (17) and/or Ia determinants on the cell surface are events which take place in the murine spleen (18). Other reports have suggested that the maturation and/or production of suppressor T cells from a population of thymic-derived precursors occurs predominantly if not exclusively within the spleen (19). Several recent reports have implicated the spleen in playing a role in regulation of immune responses. The works of Lagrange et al. and Mackaness et al. (7, 8) and of Kaplan and Streilein (9, 10) have suggested that the spleen exercises an important role during the developing inductive phases of immunologic responses to certain kinds of antigens. It is within the context of these ideas that the results reported herein must be considered.

At least with respect to the reactivity to the Y chromosome-determined antigen in C57BL/6 females, the intact normal spleen would appear to play an important role in determining the speed with which a virgin female is capable of rejecting an orthotopic male skin graft. Removal of the spleen before grafting, especially within 7 days, leads to a highly significant shortening of the MST. The results of our experiments further show that there is a population of cells in normal female spleens which is responsible for delaying the first set skin graft rejection reaction beyond 14 days. Specific immunization does not appear to be able to increase this suppressor cell activity; in fact these cells can only be identified in normal spleens and in spleens from animals up to 5 days after

exposure to a male skin graft. After 10 days, the suppressing population has disappeared. Studies currently in progress are attempting to define the nature of the cell population which is responsible for this suppressing activity and to determine whether it is a function of living cells or cell products. Our interpretation of the GVHR experiments conducted on cells from specifically sensitized and splenectomized or sham operated female donors leads us to believe that in the sham operated situation there appears to be an increase in numbers of cells capable of initiating GVH reactions. Lymphoid cell populations from animals that have been splenectomized and then immunized to male antigen appear to be devoid of cells capable of comparable reactivity.

The concept of weak and strong transplantation antigens is one that has been derived from empiric observations employing genetically defined strains of animals and observing the speed with which grafts across diverse histocompatibility barriers are rejected (20). Obviously, the speed with which an allograft of skin is rejected is a function not only of the strength of the histocompatibility antigens it bears but of the capacity of the host to respond to these antigens. The source of skin used in grafting plays a minor role in determining the MST, tail and ear skin grafts surviving approximately 4–5 days longer on average than skin from flank and dorsal body wall. Our results using body wall skin agree well with those reported by Sena et al. (21); sham splenectomy in our hands did not appreciably change the MST from 16.0 days. With regard to the Y-determined antigen, C57BL/6 females are particularly good at responding to these antigens thus incurring the destruction of isografts bearing them (15, 22). The fact that prior splenectomy shifts the balance of aggressive/suppressive forces in the direction of more rapid rejection suggests that even in first set skin graft situations, regulatory forces are brought into play which operate to suppress the response. One could interpret our results in splenectomized females to mean that weak antigens are antigens which elicit immune responses particularly susceptible to suppression and that this development is somehow intricately related to an intact normal spleen. That is, weak transplantation antigens may differ from strong transplantation antigens not only by virtue of differences in inherent immunogenicity, but by virtue of their capacity to elicit predominantly suppressor activity and that this activity interferes with and delays the development of destructive allograft immunity. That responsiveness to weak transplantation antigens such as the H-Y is controlled by immune response genes within the major histocompatibility complex is consistent with the idea (21).

Summary

The tempo with which C57BL/6 females reject male skin isografts is determined in part by the immunogenicity of the H-Y antigen and in part by the capacity of the host to respond immunologically. Our studies indicate that the spleen plays an important role in determining the briskness of the rejection process in that splenectomy 7–30 days before grafting with male skin significantly shortens the survival time. The results of reconstitution experiments suggest that a population of cells is present in spleens of normal, but not specifically sensitized, females which can restore the conventional first set reaction in splenectomized females. It is inferred that this resident population

normally operates in spleen-intact females to delay the development of specific effector responses. Lymphoid cells from H-Y antigen-sensitized, splenectomized females failed to evoke graft-vs.-host responses in males whereas similar cells from females with spleens intact did possess graft-vs.-host potential.

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