

CELLULAR REQUIREMENTS FOR THE REJECTION OF SKIN ALLOGRAFTS IN RATS*

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Cellular hypersensitivity reactions are distinguished from antibody-mediated reactions by the ability to adoptively transfer the responses from immunized to normal individuals with lymphoid cells and not serum antibody, and by the characteristic nature of the cellular infiltrate found at the affected sites. The infiltrating cells, consisting of lymphocytes and histiocytes, have been shown to be derived from a rapidly dividing precursor population (1). The majority of these cells have been found not to be specifically sensitized to the immunizing antigen (2-8) and, in delayed hypersensitivity skin reactions and autoimmune adjuvant arthritis in rats, have been traced to a bone marrow origin (9-14).

It seemed important to determine whether bone marrow cells were necessary in yet another cellular hypersensitivity response, that of skin allograft rejection. Evidence presented in this paper demonstrates that, unlike the two previously studied responses, rejection of skin allografts in rats can occur in the absence of the nonspecific marrow-derived cells. In addition, the marrow of rats is shown to contain a minority population of cells capable itself of effecting the rejection of Ag-B incompatible skin grafts.

Materials and Methods

Animals.—Male and female rats of the isogenic Lewis (Ag-B¹), BN (Ag-B³), and Fischer (Ag-B¹) strains as well as LBN F₁ hybrids were used. All animals were bred and raised in our colony.

Induction of Tolerance.—Lewis rats, less than 24 h old, were inoculated via the orbital branch of the anterior facial vein with lymphohemopoietic cells from adult donor rats. In the Ag-B incompatible BN → Lewis combination each host received 30 million bone marrow cells. Tolerance was induced in the Ag-B compatible Fischer → Lewis combination by inoculation of 1-5 million lymph node or bone marrow cells. All cell suspensions were prepared in Hanks' balanced salt solution, hereafter HBSS,¹ (Grand Island Biological Co., Grand Island, N. Y.) according to procedures described elsewhere (15) and were administered in a standard volume of 0.1 ml.

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¹ Abbreviations used in this paper: HBSS, Hanks' balanced salt solution; PPD, purified protein derivative.

Preparation of Recipients.—Rats inoculated at birth were thymectomized at 6 wk of age and grafted with skin from the appropriate strain at 7 wk (16). Only highly tolerant rats, i.e. those bearing healthy skin allografts with normal fur crops for 2–3 mo, were used as recipients in this study. At that time they were given 900 rad of total body ^{137}Ce γ -irradiation from a Gammacell 40 irradiator (Atomic Energy of Canada, Ltd., Ottawa, Ontario, Canada). Rats were held in a Lucite cage and irradiated from above and below at a constant 109 rad/min.

Sensitization of Lymph Node Donors.—

Allograft sensitivity: Lewis rats were sensitized to either BN or Fischer antigens by bilateral skin grafts followed, about 10 days later, by a booster intraperitoneal inoculation of 200–400 million lymph node and spleen cells. Rats were used as donors 7 days thereafter.

Tuberculin sensitivity: Adult Lewis rats were sensitized to tuberculin by injection of 300 μg of heat-killed tubercle bacilli suspended in oil (Bayol F; Humble Oil & Refining Co., Houston, Tex.) (0.1 ml) into one hind footpad. 9 days after sensitization lymph nodes were used in transfer experiments.

Transfer of Cells.—

Bone marrow: Normal Lewis rats, 3–4 mo of age, were killed by exsanguination and marrow removed from the humerus, tibiofibula, and femur according to the technique reported earlier (9). Dosages varying from 0.025×10^8 to 3.0×10^8 nucleated cells were injected intravenously into recipients on the day of irradiation.

Lymph node cells: Brachial, axillary, inguinal, cervical, and mesenteric nodes were removed from either normal or sensitized donors and cell suspensions prepared in HBSS (9). Tolerant recipients received 0.0025×10^8 to 2.0×10^8 viable cells intravenously on the day of irradiation.

Tuberculin Skin Tests.—All skin tests were performed by the intradermal injection of 50 μg of tuberculin purified protein derivative ([PPD] Parke, Davis & Co., Detroit, Mich.). Reactions were read at 24 h and scored by measuring the diameter of the indurated lesion (in millimeters).

Neonatal Thymectomy.—Rats, less than 24 h old, were thymectomized using the technique of Isaković et al. (17).

Treatment of Cells with Antithymus Serum.—Normal Lewis bone marrow cells were incubated with an isoantiserum directed against a marker on rat thymus and thymus-derived (T) lymphocytes (18) as follows: 1×10^7 cells suspended in 1 ml of medium were added to 1 ml of undiluted antithymus serum and incubated at 37°C for 5 min. 1 ml of guinea pig serum, diluted 1:2 with medium, was added and the mixture incubated for an additional 30 min. At the end of that time the cells were centrifuged at 180 g at 4°C for 10 min, washed with medium, and injected intravenously into recipient rats at a final dose of 10 million cells.

RESULTS

Survival of Ag-B Incompatible Skin Grafts after Transfer of Various Lymphoid Cells in Tolerant, Thymectomized, Irradiated Rats.—The first set of experiments were designed to determine whether bone marrow cells were necessary to obtain skin allograft rejection in recipients of sensitized lymph node cells. Accordingly, tolerant² Lewis rats bearing healthy BN skin grafts were thymectomized and subjected to 900 rad total body γ -irradiation followed by the transfer of Lewis anti-BN lymph node cells with or without normal marrow. A third group of rats received marrow alone. The cell dosage in these experiments was constant, each

² Because of the difficulty of getting grafted skin to heal on rats subjected to lethal total body irradiation, the rejection of healed-in skin grafts on *tolerant* hosts was studied.

recipient receiving $3-4 \times 10^8$ marrow and/or 2×10^8 lymph node cells. The results obtained (Table I) were surprising in that rats receiving lymph node cells with or without marrow cells rejected their BN skin grafts with equal intensity, quite apart from the results obtained in previous experiments with delayed hypersensitivity reactions. Recipients of marrow cells alone also rejected their skin grafts, although at a time almost twice that obtained when lymph node cells were used.

In order to determine whether the effector mechanisms of these two cellular hypersensitivity phenomena, skin allograft rejection and delayed skin reactions, were truly different in their requirements for bone marrow cells, the following experiments were performed. Tolerant, thymectomized, irradiated rats were given both anti-BN and antituberculin lymph node cells *with* or *without* normal mar-

TABLE I
Survival of BN Skin after Transfer of Marrow and/or Lymph Node Cells into Tolerant, Thymectomized, Irradiated Lewis Rats

Cells injected	No. of rats	Median survival time*
		<i>days \pm 2 SE</i>
Marrow‡ plus sensitized lymph node§	9	5.0 \pm 0.58
Sensitized lymph node§ only	5	5.5 \pm 0.18
Marrow‡ only	5	8.75 \pm 1.71

* Time after irradiation and cell transfer.

‡ 3×10^8 cells injected intravenously.

§ 4×10^8 cells injected intravenously.

row cells. 24 h after cell transfer all rats received a skin test with PPD and their reactions were read 24 h later. Skin grafts were examined daily for evidence of rejection.

The results of the experiments, seen in Table II, show a clear separation of the two cellular reactivities. Rats receiving marrow cells along with the anti-BN and tuberculin-sensitive lymphocytes (group I) produced positive tuberculin reactions and rejected their BN grafts shortly after cell transfer. On the other hand, rats that received only the lymph node cell populations and *no marrow* (group II) did not develop significant delayed skin reactions, the diameter of their reactions being not significantly different from irradiated uninjected controls (group VI). These same rats (group II) did, however, go on to reject their skin grafts. None of the rats in other groups, all lacking bone marrow cells, produced positive tuberculin reactions. However, all recipients of lymph node cells rejected their grafts promptly with the survival times between groups being not significantly different from one another ($P > 0.05$). As before, recipients of marrow cells rejected their BN grafts significantly later than rats given lymph node cells.

Rejection in irradiated reconstituted recipients was not due to any possible

TABLE II
Effect of Bone Marrow Cells on the Production of Tuberculin Reactions and Rejection of BN Skin Grafts

Group no.	Cells injected	No. of rats	Tuberculin reaction (diameter in mm) \pm 2 SE	Graft survival days \pm 2 SE
I	Marrow* plus anti-BN and Tb-sensitized lymph node†	6	10.0 \pm 1.38	4.8 \pm 0.62
II	Anti-BN and Tb-sensitized lymph node	6	6.8 \pm 1.74	5.2 \pm 0.72
III	Anti-BN-sensitized lymph node	6	5.0 \pm 0.90	5.8 \pm 0.96
IV	Tb-sensitized lymph node	5	5.0 \pm 2.82	6.0 \pm 0.82
V	Marrow only	6	4.7 \pm 2.76	9.3 \pm 2.18
VI	γ -ray only (no cells)	5	5.8 \pm 1.32	>13.0§
VII	LBN F ₁ marrow	6	—	>50
VIII	LBN F ₁ lymph node	4	—	>13§
IX	LBN F ₁ Tb-sensitized lymph node	3	—	>13.7§

* $3-4 \times 10^8$ marrow cells injected intravenously.

† 2×10^8 lymph node cells injected intravenously.

§ Time at which rats died of irradiation sickness.

nonspecific effect of irradiation or cell transfer since 900 rad γ -irradiation alone did not cause graft destruction at the time of death (group VI), nor were BN skin grafts affected by the transfer of genetically tolerant LBN F₁ hybrid cells (groups VII and VIII). In addition, the rejection of BN skin by lymph node cells from tuberculin-sensitized rats appeared not to be related to cells reactive to tuberculo-protein antigens since cells from LBN F₁ hybrid rats immunized to tuberculin did not result in graft rejection (group IX). It would appear that cells not reactive to tuberculin in the lymph node cell suspension became sensitized to BN antigens in tolerant recipients and they were the effectors of allograft rejection, inasmuch as similar numbers (2×10^8) of normal Lewis lymph node cells (Table III) resulted in the breakdown of grafts with a median survival time not different from that produced by tuberculin-sensitized cells.

Determination of Minimum Numbers of Cells for Graft Rejection.—Graded doses of normal marrow, normal lymph node, or sensitized lymph node cells were injected into recipient rats. The results (Table III) demonstrated that the median survival times of tolerated BN skin grafts after the inoculation of any single source of cells were remarkably constant within a wide range of cell numbers. There were no significant differences in the rejection times between groups until the threshold dose was reached. This threshold dose, or minimum number of cells required to obtain BN skin graft rejection, was 0.5×10^8 for normal marrow and 0.005×10^8 (500,000 cells) for both normal and sensitized lymph node cells. Despite the fact that survival times were not different over a wide range of dosages for any one cell population, significant differences between the various cells were apparent (Table IV).

TABLE III
Survival of BN Skin after Transfer of Various Dosages of Marrow or Lymph Node Cells into Tolerant, Thymectomized, Irradiated Lewis Rats

Dose of cells ($\times 10^6$)	Graft survival		
	Normal marrow	Normal lymph node	Sensitized lymph node
	<i>days \pm 2 SE</i>		
4.0	9.0 \pm 1.46 (6)*		
3.0	9.6 \pm 3.20 (5)		
2.0	8.8 \pm 1.32 (5)	6.2 \pm 0.60 (6)	5.4 \pm 0.36 (8)
1.0	9.2 \pm 1.58 (5)	7.2 \pm 1.58 (6)	5.2 \pm 0.32 (6)
0.5	10.9 \pm 1.76 (7)	7.0 \pm 1.08 (5)	6.3 \pm 0.46 (6)
0.25	10.8 \pm 1.96 (10)	7.2 \pm 0.12 (5)	6.5 \pm 0.68 (6)
0.1	9.6 \pm 2.16 (7)	6.6 \pm 0.48 (5)	5.8 \pm 0.98 (5)
0.05	8.6 \pm 1.00 (5)	7.0 \pm 0.00 (4)	6.3 \pm 0.66 (6)
0.025	15.3 \pm 1.76‡	8.4 \pm 1.00 (5)	6.2 \pm 0.74 (5)
0.01	> 11§	7.8 \pm 1.73 (4)	5.9 \pm 0.68 (8)
0.005		7.0 \pm 1.14 (4)	6.7 \pm 0.80 (9)
0.0025		> 11§	> 13

* Represents number of rats in group.

‡ Represents three survivors: $\frac{5}{8}$ died at 8-11 days with intact grafts.

§ $\frac{4}{4}$ died at 8-11 days with intact grafts.

|| $\frac{5}{5}$ died at 9-13 days with intact grafts.

TABLE IV
Median Survival Times of BN Skin Grafts after Transfer of Marrow or Lymph Node Cells

Cells injected	Dose of cells ($\times 10^6$)	No. of rats	Median survival times	P
			<i>days \pm 2 SE</i>	
Normal marrow	0.05-4.0	50	9.72 \pm 0.52	<0.001
Normal lymph node	0.005-2.0	44	7.11 \pm 0.96	
Sensitized lymph node	0.005-2.0	59	5.84 \pm 0.19	

Survival of Ag-B Compatible Skin Grafts after Transfer of Cells in Tolerant, Thymectomized, Irradiated Rats.—Lewis rats tolerant of Fischer antigens and bearing healthy Fischer skin grafts were thymectomized, lethally irradiated, and injected with Lewis lymph node cells with or without normal bone marrow cells. The results (Table V) demonstrate that, like the Ag-B incompatible system, rats that received marrow plus lymph node cells or lymph node cells alone rejected their tolerated skin grafts. However, none of the rats that received bone marrow cells alone rejected their Fischer skin grafts despite heroic dosages of up to 10×10^8 cells.

Thymus Origin of Immunocompetent Cells in Rat Bone Marrow.—To determine whether the population of cells in rat bone marrow capable of reacting to Ag-B incompatible BN antigens were thymus derived, the following experiments

TABLE V
Survival of Fischer Skin after Transfer of Marrow and/or Lymph Node Cells into Tolerant, Thymectomized, Irradiated Lewis Rats

Cells injected	Median survival time
	<i>days</i> \pm 2 SE
Marrow plus sensitized lymph node	7.0 \pm 0.0 (4)*
Sensitized lymph node only	7.6 \pm 1.28 (7)
Normal lymph node only	8.0 \pm 0.0 (4)
Marrow only (3×10^8)	> 50 (5)
Marrow only (5×10^8)	> 50 (4)
Marrow only (10×10^8)	> 50 (6)

* Represents number of rats in group.

TABLE VI
Survival of BN Skin Grafts after Transfer of Bone Marrow Cells from Normal or Neonatally Thymectomized Rats

Doses of cells ($\times 10^8$)	Marrow donors			
	Normal		Thymectomized	
	No. of rejection/total	MST (days)	No. of rejection/total	MST (days)
2.0-4.0	16/16	9.12 \pm 1.14	4/4	9.25 \pm 0.5
0.5	7/7	10.90 \pm 1.76	1/6	1 \times 44 5 \times > 50
0.25	10/10	10.80 \pm 1.96	0/3	> 50

TABLE VII
Survival of BN Skin Grafts after Transfer of Bone Marrow Cells Preincubated with Antithymus Serum

Serum incubation	Survival Times
	<i>days</i>
Antithymus serum	21, > 50, > 50
Normal serum	11, 18, 18

were performed. Marrow cells from normal or neonatally thymectomized donor rats were injected into tolerant, thymectomized, irradiated rats or normal marrow cells were first incubated with an isoantiserum against an antigenic marker on rat T cells before injection into similarly prepared recipients. The results, presented in Tables VI and VII, clearly demonstrate that these immunocompetent cells are in fact a thymus-derived population.

DISCUSSION

From the experiments reported in this paper it is evident that rejection of skin allografts in the rat can occur in the absence of nonspecific bone marrow-derived

cells. Indeed, this is in contrast to the elicitation of delayed hypersensitivity skin reactions and autoimmune adjuvant arthritis, two cellular hypersensitivity reactions that require the presence of high numbers of bone marrow cells (9, 14). These cells have been shown by immunofluorescence labeling to constitute the majority of the cells infiltrating the lesions (10, 14). In the present study lymph node cells alone, whether normal or presensitized, were capable of causing the destruction of long-standing skin grafts in a relatively short time. No difference in rejection time was apparent when normal marrow cells were transferred along with lymph node cells. Experiments in which both tuberculin reactions and skin graft rejection were studied in the same recipients showed that these two cellular immune reactivities were indeed different in their requirements for marrow cells. Only rats receiving lymph node and marrow cells produced positive delayed hypersensitivity skin reactions and rejected their BN skin grafts, whereas recipients of lymph node cells alone demonstrated only allograft rejection.

These results are in agreement with previously published work in the rabbit (19) but differ from those reported for the mouse (20). Richter et al. (19) demonstrated that irradiated rabbits rejected their skin grafts in the absence of any bone marrow cells. Unfortunately, all animals in their study rejected their grafts, even those that did not receive cells. It is difficult to reconcile the fact that 850 rad irradiated rabbits could destroy allografted skin in a time not different from untreated animals, especially in light of the early work of Dempster et al. in which doses as low as 250 rad prolonged skin graft survival (21). On the other side of the coin, Giroud et al. (20) reported the rejection of H-2 incompatible skin in irradiated mice only when marrow cells were given to these animals and not with lymph node cells. This apparent difference in the requirements for various lymphoid cell types in the rejection process is difficult to rationalize unless rabbits, rats, and mice differ in the participation of these two cell types in cellular reactivities. In at least one immunologic phenomenon a difference in requirements for marrow and lymph node cells does exist. In the rat neonatal tolerance to Ag-B incompatible skin grafts is produced consistently only by the inoculation of bone marrow cells, lymph node and spleen cells being ineffective, whereas the opposite is true for tolerance in the mouse (22).

Despite the fact that rat skin grafts can be destroyed in the presence of lymph node cells alone, this does not say that bone marrow cells may not participate in some nonspecific way in the breakdown of skin allografts in the normal situation. Certainly it has been shown that macrophages, known to be derived from a bone marrow population (23), are abundant at sites of allograft destruction (24, 25). What the present experiments do show is that the interaction of lymphocytes specifically reactive to antigens in the graft is sufficient to cause the complete destruction of that graft. It is hopeful that future experiments, utilizing the same immunofluorescence labeling technique described in the tuberculin experiments, may produce a clearer picture of the cellular events in skin graft rejection.

Although the presence of bone marrow cells, at least precursors of the non-

specific macrophages, is not imperative to obtain skin allograft rejection, it is evident that the marrow of rats contains a population of cells, themselves capable of reacting to and destroying Ag-B incompatible grafts. Tolerant, thymectomized, and lethally irradiated Lewis rats bearing Ag-B incompatible BN skin grafts rejected these grafts when inoculated with normal Lewis marrow cells. These cells were incapable, however, of reacting to minor histocompatibility antigens distinguishing the Ag-B compatible Lewis and Fischer strains. Even high cell doses of 10×10^8 Lewis marrow were ineffective in causing the destruction of healed-in Fischer skin grafts. Either cells reactive to these minor transplantation antigens are absent in bone marrow or are present in insufficient numbers to cause graft rejection.

Evidence from two groups of experiments reported here indicates that the reactive cells in rat bone marrow are thymus-derived or T cells. The numbers of these cells seem greatly reduced in the marrow of neonatally thymectomized Lewis rats. Recipients of very high doses (2×10^8) of marrow cells from thymectomized donors rejected their grafts in a time not significantly different from recipients of normal marrow. On the other hand, rats receiving 0.25×10^8 or 0.5×10^8 bone marrow cells from thymectomized donors maintained healthy skin grafts indefinitely, whereas equivalent doses of normal marrow resulted in prompt skin graft rejection. In addition, marrow cells treated with an antiserum directed against a rat T cell antigen before inoculation into irradiated recipients were unable to react to and destroy BN skin grafts.

Evidence from a number of other laboratories have shown that both rat and mouse bone marrow contains a population of cells, probably thymus derived, capable of reacting to a variety of antigenic stimuli. These include the *in vitro* cytotoxic action against *H-2* transplantation antigens (26), ability to induce graft-vs.-host disease (27), and the production of antisheep red blood cell antibodies (28). In both the mouse (26) and rat (28) the numbers of competent cells in the marrow have been shown to vary from strain to strain.

It is apparent that very few cells are necessary to obtain rejection of Ag-B incompatible skin. As few as 500,000 lymph node cells, either normal or presensitized, can effect the prompt destruction of a healed-in graft. Although the survival times of BN grafts, with all effective doses, were significantly less for sensitized cells than normal cells, the numbers of cells required were identical for both populations. However, the minimum numbers of bone marrow cells required were found to be 10 times that of lymph node cells. This difference between marrow and lymph node cells seems to reflect the level of thymus-derived cells. Experiments utilizing antirat thymus serum have shown that 60.9% of lymph node cells and only 6.1% of bone marrow cells are susceptible to its action (18), again a 10-fold difference. This is in agreement with the estimated numbers of recirculating lymphocytes found in rat bone marrow.³

³ Scott, D. W., unpublished data.

It may be noted that tolerant, irradiated recipients of lymph node cells rejected their grafts extremely fast from the time of transfer. This is particularly true for recipients of sensitized cells where grafts were destroyed in less than 6 days. Although it has been difficult to obtain data on second-set survival times in the rat, particularly in Ag-B incompatible combinations (first set for BN \rightarrow Lewis = 8.01 days), the rapid rejection times reported here would certainly be faster than expected. This may well be due to the fact that transferred sensitized cells constitute a higher percentage of the total peripheral lymphocyte population in lethally irradiated recipients. Similar results were reported for delayed hypersensitivity reactions in irradiated rats (9). Another possible factor, more related to graft rejection by recipients of normal cells, would be release of antigen from the cells of the healed-in BN graft as a result of irradiation damage. This would allow for easier access of immunologically competent cells to such antigens and result in a more rapid sensitization.

SUMMARY

The role of bone marrow-derived cells in the rejection of skin allografts in rats was investigated. Lewis rats, rendered tolerant of BN antigens and bearing healthy grafts, were thymectomized, irradiated with 900 rad, and injected with varying doses of either normal isologous bone marrow, normal lymph node cells, and/or lymph node cells presensitized to BN antigens. In some experiments rats were also adoptively sensitized to tuberculin. Results showed that, although necessary for the elicitation of tuberculin skin reactions, bone marrow cells are not needed for the rejection of previously tolerated skin allografts. Rats receiving lymph node cells alone rejected their grafts in about 6–7 days.

In addition, rats injected with bone marrow alone also rejected their grafts, although significantly later than did lymph node cell recipients, indicating that rat marrow contains a population of cells capable of reacting to transplantation antigens. These cells were found capable of reacting to major transplantation antigens but not minor as they were ineffective in causing the rejection of Ag-B compatible Fischer skin grafts. From experiments utilizing bone marrow from neonatally thymectomized donors and cells treated with an antiserum to rat T cells, these competent cells in the marrow were shown to be thymus derived.

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