THE INDUCTION OF GRAFT VERSUS HOST DISEASE IN MICE TREATED WITH CYCLOPHOSPHAMIDE*

By ALBERT H. OWENS, Jr., M.D., and GEORGE W. SANTOS, M.D.

(From the Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, and the Oncology Service, Baltimore City Hospitals, Baltimore, Maryland 21224)

(Received for publication 28 March 1968)

Graft versus host reactions have been encountered in several situations in which recipient animals have been unable to defend themselves against grafts of immunologically competent foreign cells. Such reactions follow the grafting of genetically different adult lymphoid cells to embryos and newborns and have been termed "runting disease" (1–5). A graft versus host syndrome can be induced in adult F_1 hybrid animals by the administration of large numbers of lymphoid cells from either of its homozygous parent strains (6, 7). Similar evidences of an immunologic attack by either parent on F_1 hybrids have been shown following parabiotic union (8, 9). Further, graft versus host disease follows the transfer of foreign immune competent cells to adult animals previously treated with lethal doses of whole body irradiation (10, 11) or who had been thymectomized shortly after birth (12).

In the present studies the occurrence of a graft versus host disease is described in a somewhat different experimental system. An acute immunologic reaction against adult F_1 hybrid mice is shown to follow the grafting of a variety of parental, allogeneic, and xenogeneic immunologically responsive cells to cyclophosphamide-treated recipients.

Materials and Methods

Animals.[1]—Female BDF_1 (C57BL/6 × DBA/2) mice, age 10–12 wk, were used as recipients. Donor mice of similar sex and age were chosen from the C57BL/6, DBA/2, C3H, and BALB/c strains. Rat bone marrow cells were obtained from Sprague-Dawley females, age 10–12 wk. All animals were housed in covered plastic cages, 10 or fewer per cage, and provided with tap water and a standard laboratory diet[2] ad lib.

Drug.[3]—Cyclophosphamide (CY) and mechlorethamine (HN2) were dissolved in saline and used within 10 min.

* These studies were supported by Public Health Service Grants CA-6973 and CA-5091, and Research Career Development Award K3-CA-25,344.

The work was previously presented in part in 1963. *Federation Proc.* **22**:393.

[1] Inbred mice were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me., and Microbiologic Associates, Inc., Bethesda, Md.

[2] Purina Chow Checkers.

[3] Drugs were supplied generously by the Cancer Chemotherapy National Service Center.

Cell Preparations.—Peripheral blood was collected by bleeding from the retro-orbital venous plexus under light ether anesthesia. The nucleated leukocytes were separated, washed, and concentrated in cold Tyrode's solution as described previously (13).

Donor animals for other cells were killed by cervical dislocation. Spleens were minced with a fine scissors and gently homogenized with a loose fitting glass TenBroeck homogenizer. The crude spleen cell suspension was filtered through surgical gauze, washed, and finally suspended in cold Tyrode's solution (13). Thymus tissue was dissected from the mediastinum and cell suspensions were prepared as from spleens. Bone marrow was obtained from the femurs. The pulpy marrow was dissociated by gentle agitation, filtered through surgical gauze, washed, and the cells finally suspended in cold Tyrode's solution (13).

Nucleated cell suspensions were counted with a standard clinical hemocytometer. Trypan blue dye exclusion was used to assess the number of "viable" cells (13, 14). Peripheral blood cell suspensions were 3–5% trypan blue positive, bone marrow suspensions 10–15% positive, and spleen and thymus cell preparations 20–25% positive. Cell suspensions were injected into a tail vein in a volume of 0.5 ml except at the highest cell dose levels, where 1.0 ml was used. All cell doses were calculated as viable cells.

Wright's stain was used to distinguish morphologic cell types. The alkaline phosphatase stain was used to identify rat polymorphonuclear leukocytes (10, 15).

Basic Experimental Protocol.—Prospective recipient mice were treated with graded single doses of cyclophosphamide or mechlorethamine injected intraperitoneally on day 0. 24 hr later (day 1), donor cell suspensions were administered slowly by a single intravenous injection. The mice were observed daily for a 60 day period and weighed three times weekly.

This study is based on several series of experiments. In each series a single experimental variable was examined and its controls were evaluated concurrently. Each series of studies was repeated at least two or three times and, unless otherwise noted, final statistical analysis was not completed until each experimental point comprised 30–40 mice.

Experimental controls were employed as indicated. At times, isotonic saline was used in place of cyclophosphamide or mechlorethamine. The several controls used in place of donor cell suspensions included isotonic saline, cell preparations lysed in hypotonic aqueous solution and cell suspensions heated at 56°C for 20 min. In some experiments viable isogeneic cells were given in place of similar foreign cells.

Statistical Analysis.[4]—All regression lines were fitted by the method of least squares and their slopes determined. Statistical methodology is based on standard texts and has been described previously (16). The method of Berkson (17) was used to calculate the LD_{50} values.

RESULTS

Mice given cyclophosphamide and mature foreign nucleated cells rapidly developed a characteristic clinical illness which resembled the graft versus host disease encountered in studies of radiation chimeras and in F_1 hybrid mice who had received large doses of parental lymphoid cells. Within 5–10 days of receiving the foreign cells, the affected animals became very lethargic and assumed a hunched posture. Their fur was ruffled and unkempt and their faces were often edematous. Crusted secretions frequently formed about the eyes and perianal area. These mice moved about with a peculiar, unsteady, high-stepping gait. Severe wasting developed, animals often losing more than 25% of their initial

[4] Analysis of the data was facilitated by the Computing Center of the Johns Hopkins Medical Institutions which is supported by United States Public Health Service Grant FR-00004.

body weight. Although the stools were sometimes soft and sticky, diarrhea was not a prominent feature of this syndrome. Neither did a clinically evident generalized dermatitis develop with any characteristic frequency.

The more severely affected animals began to die within 5–7 days and deaths continued to occur through days 25–30. Only rarely were additional deaths observed between days 30 and 60. Mice developing a milder form of the disorder recovered spontaneously, usually within the 3rd–6th wk of observation. At autopsy, the severely wasted animals appeared pale, but not icteric. The thymus and lymph nodes were unusually small. The spleen was of variable size, enlarged in the mice dying earlier in the course of the illness and small in animals dying later. In general, histologic study revealed tissue changes similar to those reported in other types of graft versus host disease.

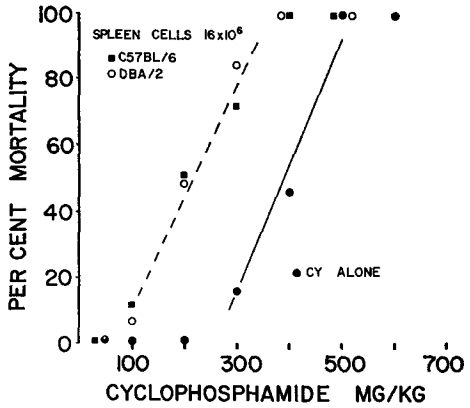

FIG. 1. The effect of cyclophosphamide dosage on mortality due to graft versus host disease. The fate of drug-treated mice given 16×10^6 spleen cells from C57BL/6 or DBA/2 donors is compared with that of mice given graded doses of cyclophosphamide alone.

Effect of Drug Treatment.—Several experiments were performed in which groups of BDF_1 mice were given graded single doses of cyclophosphamide ranging from 50 to 600 mg per kg. 24 hr later they received a graft of 16×10^6 nucleated spleen cells from the C57BL/6, DBA/2, or C3H strains. Other BDF_1 mice were given similar graded doses of cyclophosphamide, but saline instead of spleen cells.

When compared with animals treated with cyclophosphamide and saline, an increased mortality was observed in those groups given cyclophosphamide and parental spleen cells (Fig. 1). Similar results were encountered in mice given cyclophosphamide and C3H spleen cells. The clinical course of the animals developing the wasting syndrome as described previously could be distinguished from the effects caused by cyclophosphamide alone (Table I). A positive dose-response relationship existed between the level of the cyclophosphamide dose

and the severity of this wasting syndrome. Mice given isotonic saline in place of cyclophosphamide developed no apparent illness when given 16 × 10^6 C57BL/6, DBA/2, or C3H spleen cells 1 day later.

In a similar series of experiments, BDF_1 mice were given graded single doses of mechlorethamine ranging from 2 to 16 mg per kg. 24 hr later they received

TABLE I

The Effect of Parental and Allogeneic Spleen Cells on Mortality Caused by Graded Doses of Cyclophosphamide

Donor strain (16 × 10^6 cells)	Mortality*		
	LD_{10} ± SE	LD_{50} ± SE	LD_{90} ± SE
O	278 ± 28	411 ± 26	608 ± 109
C57BL/6	95 ± 17	210 ± 16	463 ± 84
DBA/2	111 ± 13	196 ± 12	343 ± 37
C3H	98 ± 16	213 ± 18	423 ± 64

* Dose of cyclophosphamide (mg per kg) required to kill 10, 50, and 90% of mice.

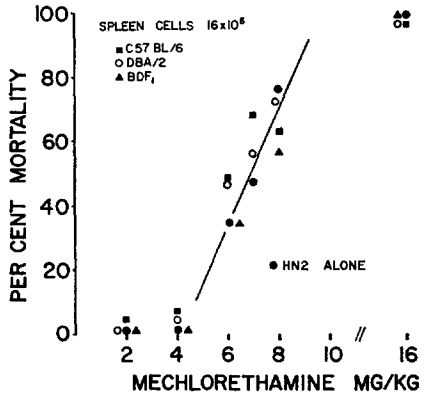

FIG. 2. The effect of mechlorethamine on the induction of graft versus host disease. The fate of drug-treated mice given 16 × 10^6 spleen cells from C57BL/6, DBA/2, and BDF_1 donors is the same as those given graded doses of mechlorethamine alone.

16 × 10^6 nucleated spleen cells from C57BL/6, or DBA/2 donors. Additional groups of BDF_1 mice were given identical graded doses of mechlorethamine, but saline in place of spleen cells. The clinical course and mortality encountered in mice receiving mechlorethamine and spleen cells could not be distinguished from comparable groups given drug alone (Fig. 2). No stigmata of the graft versus host reaction were apparent.

Effect of Parental Cells.—In a second series of experiments several groups of

BDF_1 mice were treated with a single dose of cyclophosphamide, 300 mg per kg. 24 hr later these mice were given graded doses of spleen cells ranging from 4 to 32×10^6 nucleated cells. Donor animals were from either parental strain, the C57BL/6, or DBA/2.

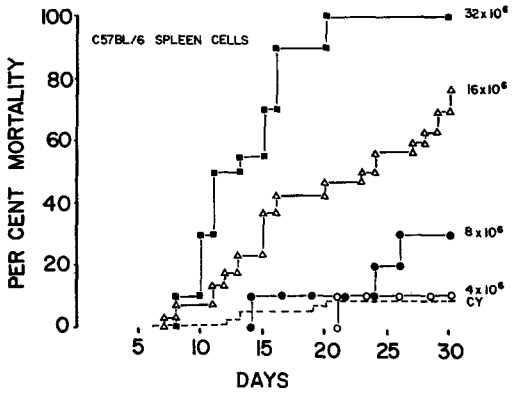

FIG. 3. The effect of C57BL/6 spleen cells on the induction of graft versus host disease The fate of drug-treated mice given graded doses of cells is compared with those receiving cyclophosphamide, 300 mg per kg, alone.

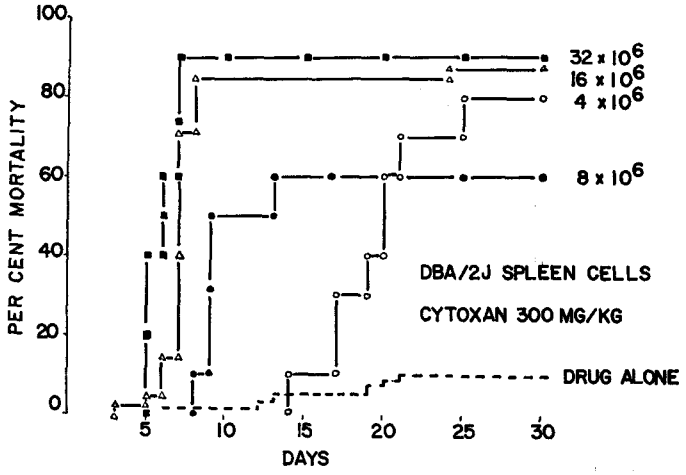

FIG. 4. The effect of DBA/2 spleen cells on the induction of graft versus host disease. The fate of drug-treated mice given graded doses of cells is compared with those receiving cyclophosphamide, 300 mg per kg, alone.

The severe wasting of the graft versus host reaction was apparent in the mice treated with cyclophosphamide and spleen cells and could be distinguished from the effects of cyclophosphamide alone. The cumulative mortality caused by this rather acute graft versus host reaction is shown in Figs. 3 and 4. These data

also indicate a positive relationship between the size of the spleen cell inoculum and the resultant severity of the graft versus host disease. Cells derived from either parental strain produced similar results (Table II). Although DBA/2 spleen cells would appear to be six or seven times more potent than those from

TABLE II

Mortality Due to Graded Doses of Isogeneic, Parental, and Allogeneic Spleen Cells in Mice Pretreated with Cyclophosphamide

Donor strain ($4–64 \times 10^6$ cells)	Mortality*		
	$LD_{10} \pm SE$	$LD_{50} \pm SE$	$LD_{90} \pm SE$
BDF_1	No dose effect seen		
C57BL/6	4.4 ± 0.65	10.0 ± 0.8	22.5 ± 4.0
DBA/2	0.08 ± 0.2	1.5 ± 1.3	31 ± 20
C3H	0.5 ± 0.4	4.0 ± 1.1	32 ± 12
BALB/c	1.6 ± 0.6	7.9 ± 1.0	38 ± 13

* Number of cells 10^6 required to kill 10, 50, and 90% of mice.

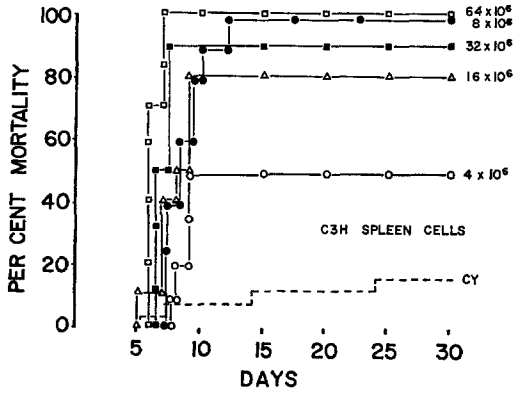

FIG. 5. The effect of C3H spleen cells on the induction of graft versus host disease. The fate of drug-treated mice given graded doses of cells is compared with those receiving cyclophosphamide, 300 mg per kg, alone.

the C57BL/6, such a comparison was not thought trustworthy since interstrain experiments were not performed concurrently.

Graft versus host disease did not occur when animals were given lysed or heated spleen cells. Neither was it seen in mice given 32×10^6 nucleated spleen cells from either parent in the absence of cyclophosphamide pretreatment.

Effect of Allogeneic Cells.—In a third series of experiments groups of BDF_1 mice were given a single dose of cyclophosphamide, 300 mg per kg, 24 hr before graded inocula of allogeneic spleen cells. The dose levels of spleen cells ranged

from 4 to 64 × 10^6 nucleated cells. Donor animals were from the C3H and BALB/c strains.

Animals treated with cyclophosphamide and "viable" spleen cells developed the characteristic clinical manifestations of the graft versus host reaction. The resultant illness was more severe in those mice given the larger doses of spleen cells (Figs. 5 and 6). One could not distinguish clearly between the consequences of spleen cell grafts from the C3H or BALB/c strains (Table II).

No clinical evidence of the graft versus host reaction was detected in the animals treated with cyclophosphamide alone. Neither was this syndrome seen in mice given 64 × 10^6 C3H or BALB/c spleen cells without drug pretreatment.

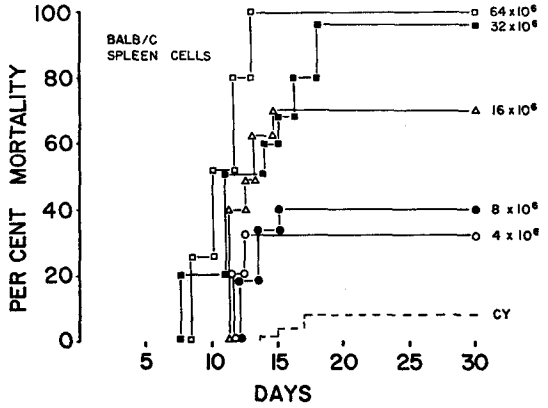

FIG. 6. The effect of BALB/c spleen cells on the induction of graft versus host disease. The fate of drug-treated mice given graded doses of cells is compared with those receiving cyclophosphamide, 300 mg per kg, alone.

Effect of Xenogeneic Cells.—The graft versus host disease also developed in BDF_1 mice treated with cyclophosphamide, 300 mg per kg, and given rat bone marrow, 70 or 140 × 10^6 viable nucleated cells, 24 hr later. A clear-cut but less severe wasting syndrome occurred in animals pretreated with a smaller dose of cyclophosphamide, 200 mg per kg, prior to grafting (Table III). Although allogeneic mouse marrow cells (Fig. 7) appear to be four to five times more capable of inducing graft versus host disease than cells from rat marrow when given to recipients pretreated with cyclophosphamide, 300 mg per kg, such a comparison would not seem valid since the interspecies comparisons were not completed concurrently.

When recipient mice were pretreated with mechlorethamine, 6 mg per kg or given isotonic saline 24 hr before the rat marrow grafts, no evidence of the graft versus host reaction developed. Further, this type of wasting syndrome was not

seen following the administration of rat bone marrow, 140 × 10^6 nucleated cells, without cyclophosphamide pretreatment (Table III).

In a similar series of experiments, BDF_1 mice given cyclophosphamide or mechlorethamine and rat bone marrow cells were examined for the persistence

TABLE III

Mortality Encountered in Mice Treated with Cyclophosphamide or Mechlorethamine and Rat Bone Marrow

Pretreatment drug	Bone marrow (nucleated cells)	No. mice	Mortality
mg/kg			%
Cyclophosphamide			
300	140 × 10^6	12	100
300	70 × 10^6	9	78
300	0	10	20
200	140 × 10^6	13	46
200	70 × 10^6	10	50
200	0	10	0
Mechlorethamine			
6	140 × 10^6	13	23
6	70 × 10^6	9	33
6	0	10	30
None	140 × 10^6	8	0

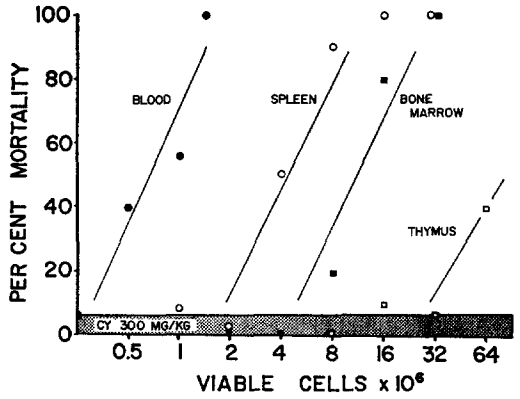

FIG. 7. A comparison of the fate of drug-treated mice given graded doses of nucleated cells from the peripheral blood, spleen, bone marrow, and thymus of C57BL/6 donors and those receiving cyclophosphamide, 300 mg per kg, alone.

of rat polymorphonuclear leukocytes in their peripheral blood, spleen, and bone marrow. The alkaline phosphatase staining reaction was used to distinguish the presence of rat granulocytes, since mouse cells are not stained by this technique (11, 15).

It would appear that rat polymorphonuclear leukocytes were promptly disposed of by recipient mice who were given no drug pretreatment or by those who received mechlorethamine (Table IV). In none of these animals could alkaline phosphatase–positive granulocytes be detected in the blood, spleen, or marrow 48 hr after the graft. In contrast, in mice pretreated with cyclophosphamide, alkaline phosphatase–positive cells were detected for at least 14 days. These foreign cells more frequently persisted in animals treated with the higher dose of cyclophosphamide and given the larger inoculum of rat bone marrow.

Effects of Grafts from Various Tissues.—The ability of cells from various foreign tissues to induce the graft versus host syndrome was examined in a fifth set of experiments. In this series recipient BDF_1 mice were treated with a

TABLE IV

Persistence of Rat Polymorphonuclear Leukocytes in the Tissues of Mice Given Cyclophosphamide or Mechlorethamine and Rat Bone Marrow

Pretreatment drug	Rat bone marrow (nucleated cells)	Persistence of rat cells (days after transfer)			
		2	6	10	14
mg/kg					
Cyclophosphamide					
300	140×10^6	9/9*	7/8	8/9	6/6
300	70×10^6	9/9	6/10	1/7	0/9
300	0	0/10			
200	140×10^6		2/10	1/6	
Mechlorethamine					
6	140×10^6	0/11			
6	0	0/10			
None	140×10^6	0/10			

* Number of mice with alkaline phosphatase–positive granulocytes per number of mice examined.

single dose of cyclophosphamide, 300 mg per kg, 24 hr prior to receiving a suspension of cells from C57BL/6 (parental) donors. Graded inocula of cells were prepared from the peripheral blood (range $0.25–1.5 \times 10^6$), spleen (range $1–32 \times 10^6$), bone marrow (range $2–32 \times 10^6$), and thymus (range $8–64 \times 10^6$).

The characteristic clinical manifestations of the graft versus host reaction were noted in all experimental groups given cyclophosphamide and inocula of viable nucleated cells. The mortality due to this immunologic disorder could be readily related to the size of the foreign cell graft (Fig. 7). It would appear that cells from the peripheral blood were most capable of inducing the graft versus host disease and those from the thymus least effective. Cells from the spleen and bone marrow were of intermediate potency, the spleen cells appearing somewhat more effective (Table V). Since the thymus cell suspensions were not visibly hemoglobin stained, it seems unlikely that their effect resulted solely from contamination by peripheral blood (13).

Experimental Controls.—In each series of experiments a number of controls were used in place of drug treatment or the grafts of viable cells. They may be listed as follows:

1. Isotonic saline was substituted for cyclophosphamide or mechlorethamine pretreatment in animals being given the largest dose of nucleated cells under evaluation (cells alone control).
2. Equivalent cell preparations, previously lysed by water, were given in place of the largest inoculum of cells being studied (lysed cell control).
3. Cell suspensions equal to the largest inoculum being studied were heat-killed prior to injection in place of viable cells (heat-killed cell control).
4. Isotonic saline was substituted also for the cell suspension in each series of trials (drug alone control).

TABLE V

Mortality Due to Graded Doses of Parental Cells in Mice Pretreated with Cyclophosphamide

Tissue source (C57BL/6 donor)	Mortality*		
	$LD_{10} \pm SE$	$LD_{50} \pm SE$	$LD_{90} \pm SE$
Blood	0.2 ± 0.06	0.8 ± 0.09	2.6 ± 1.0
Spleen	2.0 ± 0.3	4.0 ± 0.3	7.9 ± 1.2
Marrow	6.6 ± 1.0	11.3 ± 0.9	19 ± 2.8
Thymus	32 ± 8	84 ± 9	217 ± 58

* Number of cells 10^6 required to kill 10, 50, and 90% of mice.

In none of these control groups were evidences of the graft versus host disease observed.

Additional studies were completed in mice given isogeneic (BDF_1) cells equivalent in tissue type and amount to the foreign grafts being used. In no instance did the severe wasting syndrome develop in animals given isogeneic cells from the peripheral blood, spleen, bone marrow, or thymus. Nor could it could it be shown that isogeneic cells derived from the spleen or bone marrow and given in inocula of 4–64 × 10^6 nucleated cells 24 hr after cyclophosphamide or mechlorethamine altered the mortality caused by these drugs (e.g., Fig. 2).

DISCUSSION

Graft versus host reactions have been observed in several species, including man (10, 11, 18). They occur when recipient animals are unable to reject grafts of foreign immunologically competent cells due to immunologic immaturity, genetic unresponsiveness, or prior immunosuppressive treatment with whole body irradiation. The work of Billingham and Brent (1, 2), and Simonsen (4) in newborn and embryo mice and chickens did much to establish the immunologic nature of this disorder. For example, from their studies of the "runting disease"

caused by the grafting of adult spleen cells of various allogeneic strains to newborn mice, Billingham and Brent concluded that "runting" resulted from an immunologic attack of donor immunoresponsive cells on the recipient. Working with adult mice, Vos et al. (6) and Oliner et al. (7) added to this concept by showing that a similar wasting disorder developed in F_1 hybrid recipients grafted with large doses ($> 10^8$ nucleated cells) of immunocompetent cells from either parent. Trentin (8) and van Bekkum (9) identified an analogous disorder in the F_1 hybrid partner following parabiotic union with a mouse of a parental strain. More recently, Aisenberg et al. (12) described the induction of graft versus host disease in adult rats who had been thymectomized as newborns and inoculated with foreign spleen cells when 9–13 wk of age. In addition, numerous instances of graft versus host disease have been documented in a variety of animal species when foreign bone marrow of lymphoid tissue is grafted to adult recipients previously treated with whole body irradiation (10, 11).

The acute wasting syndrome described in the present report occurred in adult mice treated with cyclophosphamide and modest inocula of foreign immunologically competent cells. Outwardly it strongly resembled the graft versus host disorders previously described in adult animals, especially the "acute killing killing effect" noted in irradiated animals given allogeneic bone marrow and lymph node cells (6, 11, 19, 20). The gross autopsy findings of pallor, thymus and lymph node atrophy, and a variable enlargement or atrophy of the spleen were also similar. We have also noted analogous clinical phenomena in rats treated with cyclophosphamide and foreign cells (21). The major findings encountered in the current studies could be distinguished from the effects caused by drug alone.

Cyclophosphamide pretreatment is required for the induction of this variety of graft versus host disease and its effectiveness in this regard is dose related. In contrast, mechlorethamine given in comparable doses is ineffective. It is of interest that sublethal doses of cyclophosphamide are sufficient for the induction of graft versus host disease. In general, higher doses (in terms of their relative lethality) of whole body irradiation are required when such small inocula of foreign cells are given (10, 11).

Cyclophosphamide has been shown to be a potent immunosuppressive drug in several species (22) and may be used to inhibit the formation of humoral antibody and prolong the survival of foreign skin and tumor grafts in rodents (23–30). Further, the present experiments indicate that cyclophosphamide can inhibit the rejection of rat granulocytes by mice. When carefully compared with cyclophosphamide, mechlorethamine has been found to be a less effective immunosuppressant (22). Indeed, in rats and mice mechlorethamine failed to suppress the primary antibody response to sheep erythrocytes even when given in lethal doses (29, 30). Similarly, in the current studies, mechlorethamine was unable to inhibit the rejection of rat granulocytes when used at an LD_{25} level.

While it seems logical to relate the immunosuppressive properties of cyclo-

phosphamide to its effectiveness in facilitating the induction of graft versus host disease following inoculations of allogeneic or xenogeneic cells, this argument does not seem appropriate to the similar syndrome which follows the grafting of homozygous parental cells to the F_1 hybrid where there is genetic tolerance of donor cells by the host. Despite this genetic tolerance, modest numbers of immunologically competent cells (up to 10^6 or 10^7) from the parent cannot cause a lethal graft versus host disease in normal adult hybrid mice whereas they can in newborn or embryo hybrid recipients (18). In studies of radiation chimeras, workers have visualized whole body irradiation as providing "space" in the adult hybrid recipient for parental cells to lodge and prolierate and thus give rise to graft versus host disease (31, 32). The nature of this space or poliferative stimulus is not clear, but it is not thought by most investigators to be an immunologic phenomenon.

With this in mind, the contrasting effectiveness of cyclophosphamide and mechlorethamine in the induction of graft versus host disease in the current studies involving parent to hybrid grafts is interesting to contemplate. Both alkylating agents when given at the dose levels used in these studies cause a prompt lymphopenia and a marked decrease in the size of the lymph nodes, spleen, and thymus. Thus, one might expect that both drugs would be effective in the induction of graft versus host disease since they both provide a great deal of "space" within the lymphoid tissues of the recipient animals.

The presence of distinctive "recessive" antigens in the homozygous cells would provide one possible explanation linking the immunosuppressive characteristics of cyclophosphamide to its role in the induction of graft versus host disease in adult hybrid mice given cells from either parent. Cudkowicz (33) has furnished data which suggest the existence of recessive transplantation antigens. His findings, however, have not been confirmed by other investigators. Further, the report (34) that adult BALB/c mice treated with cyclophosphamide and (BALB/c × DBA)F_1 spleen cells did not develop graft versus host disease despite the establishment of chimerism does not support the existence of recessive antigens in parental cells.

A second requirement for the induction of this type of graft versus host disorder is the grafting of genetically dissimilar immunoresponsive cells. This has been demonstrated by the ability of viable parental (C57BL/6 and DBA/2), allogeneic (C3H and BALB/c), and xenogeneic (Sprague-Dawley rat) peripheral blood, spleen, bone marrow, and thymus cells to evoke this disorder. An analogous wasting disease did not develop when cyclophosphamide-treated mice were given saline, lysed, or heat-killed foreign cells in place of similar viable ones. Neither did comparable inocula of viable isogeneic (BDF_1) cells initiate the disease.

In all experiments employing graded doses of viable foreign spleen cells, a reasonable dose-response relationship was observed between the size of the cell

inoculum and the resultant severity of the graft versus host disorder. The larger cell inocula resulted in a more severe disease. In the studies using graded doses of viable parental (C57BL/6) cells obtained from the peripheral blood, spleen, bone marrow, and thymus, a similar dose-response relationship was encountered. Further, it was found that cell suspensions from the blood were the most potent inducers of the wasting syndrome. Thymus cell inocula were least effective and bone marrow and spleen cells increasingly more potent in that order. It is of interest that the relative potency of these tissues to initiate graft versus host disease is analogous to their ability to produce humoral antibody when adoptively transferred to cyclophosphamide-treated mice (13) and to their ability to induce tolerance and runt disease in neonatal mice (35). Similarly, it has been shown that foreign lymphoid cells are more effective than comparable numbers of cells from bone marrow in initiating graft versus host disease in lethally X-irradiated recipient animals (6, 11, 19, 20).

The prolonged persistence of rat granulocytes in cyclophosphamide-pretreated host mice suggests that it is possible for other types of foreign cells to survive including those capable of immunologic responsiveness. Indeed, we have documented prolonged cyclophosphamide-induced lymphoid chimerism in mice (36) as have Glynn et al. (34). Further we have proven the presence of long-term lymphoid and hematopoietic chimerism in rats treated with lethal doses of cyclophosphamide and allogeneic bone marrow (37).

SUMMARY

In these studies adult mice treated with cyclophosphamide and foreign immunologically competent cells developed a graft versus host disease which outwardly resembled that encountered in other experimental systems. Progressively larger doses of cyclophosphamide produced an increasingly severe disease whereas comparable doses of mechlorethamine were ineffective. Increasingly larger cell inocula from parental, allogeneic, and xenogeneic donors resulted in a correspondingly more severe disease. Nucleated cells obtained from the peripheral blood were found to be the most potent inducers of this syndrome, while cells from the spleen, bone marrow, and thymus displayed lesser degrees of reactivity in that order. No such graft versus host disease occurred in mice given saline, lysed, or heat-killed cells in place of viable foreign cells. Neither did the disorder develop when comparable inocula of isogeneic cells were used.

The authors wish to gratefully acknowledge the excellent technical assistance of Miss Ruth Angel, Miss Priscilla Ramos, and Mrs. Margaret Bauersfeld.

BIBLIOGRAPHY

1. Billingham, R. E., and L. Brent. 1967. A simple method for inducing tolerance of skin homografts in mice. *Transplant. Bull.* **4**:67.

2. Billingham, R. E., and L. Brent. 1959. Quantitative studies on tissue transplantation immunity. IV. Induction of tolerance in newborn mice and studies on the phenomenon of runt disease. *Phil. Trans. Roy. Soc. London, Ser. B.* **242**:439.
3. Woodruff, M. F. A., and M. Sparrow. 1957. Further observations on the induction of tolerance of skin homograft in rats. *Transplant. Bull.* **4**:157.
4. Simonsen, M. 1957. The impact on the developing embryo and newborn animal of adult homologous cells. *Acta. Pathol. Microbiol. Scand.* **40**:480.
5. Porter, K. A. 1960. Immune hemolysis: a feature of secondary disease and runt disease in the rabbit. *Ann. N. Y. Acad. Sci.* **87**:391.
6. Vos, O., M. J. de Vries, J. C. Collenteur, and D. W. van Bekkum. 1959. Transplantation of homologous and heterologous lymphoid cells in x-irradiated and non-irradiated mice. *J. Natl. Cancer Inst.* **23**:53.
7. Oliner,H., R. Schwartz, and W. Dameshek. 1961. Studies in experimental autoimmune disorders. I. Clinical and laboratory features of autoimmunization (runt disease) in the mouse. *Blood.* **17**:20.
8. Trentin, J. J. 1959. Homologous disease in unirradiated F_1 hybrid mice receiving parental lymphoid tissue. *In* Biologic Problems of Grafting. F. Albert, and G. Lejeune-Ledant, editors. Blackwell Scientific Publications, Oxford. 207.
9. van Bekkum, D. W., O. Vos, and W. W. H. Weyzen. 1959. The pathogenesis of the secondary disease after foreign bone marrow transplantation in x-irradiated mice. *J. Natl. Cancer Inst.* **23**:75.
10. Koller, P. C., A. J. S. Davies, and S. M. H. Doak. 1961. Radiation chimeras. *Advan. Cancer Res.* **6**:181.
11. van Bekkum, D. W., and M. J. de Vries. 1967. Radiation Chimeras. Logos Press, London.
12. Aisenberg, A. C., B. Wilkes, and B. H. Waksman. 1962. The production of runt disease in rats thymectomized at birth. *J. Exptl. Med.* **116**:759.
13. Santos, G. W., and A. H. Owens, Jr. 1966. Adoptive transfer of immunologically competent cells. II. Quantitative studies of antibody formation by various syngeneic mouse tissues. *Bull. Johns Hopkins Hosp.* **118**:127.
14. Boyse, E. A., L. J. Old, and I. Chouroulinkov. 1964. Cytotoxic test for demonstration of mouse antibody. *In* Methods in Medical Research. H. N. Eisen, editor. Yearbook Medical Publishers, Inc., Chicago. **10**:39.
15. Nowell, P. C., L. J. Cole, J. G. Habermeyer, and P. L. Roan. 1956. Growth and continued function of rat marrow cells in x-radiated mice. *Cancer Res.* **16**:258.
16. Santos, G. W., and A. H. Owens, Jr. 1966. Adoptive transfer of immunologically competent cells. I. Quantitative studies of antibody formation by syngeneic spleen cells in the cylophosphamide pretreated mouse. *Bull. Johns Hopkins Hosp.* **118**:109.
17. Berkson, J. 1953. A statistically precise and relatively simple method of estimating the bio-assay with quantal response, based on the logistic function. *J. Am. Statis. Assoc.* **48**:565.
18. Simonsen, M. 1962. Graft versus host reactions. Their natural history, and applicability as tools of research. *Progr. Allergy.* **6**:349.
19. Schwartz, E. E., A. C. Upton, and C. C. Congdon. 1957. A fatal reaction caused by implantation of adult parental spleen tissue in irradiated F_1 mice. *Proc. Soc. Exptl. Biol. Med.* **96**:797.

20. Makinodan, T., N. Gengozian, and I. C. Shekarchi. 1958. Relative effects of splenic and bone-marrow cells in lethally irradiated mice. *J. Natl. Cancer Inst.* **20**:591.
21. Santos, G. W., and A. H. Owens, Jr. 1966. Production of graft versus host disease in the rat and its treatment with cytotoxic agents. *Nature.* **210**:139.
22. Santos, G. W. 1967. Immunosuppressive drugs. I. *Federation Proc.* **26**:907.
23. Berenbaum, M. C. 1962. A screen for agents inhibiting the immune response and the growth of tumours. *Nature.* **196**:384.
24. Berenbaum, M. C. 1963. Prolongation of homograft survival in mice with single doses of cyclophosphamide. *Nature.* **200**:84.
25. Fox, M. 1964. Suppression of tissue immunity by cyclophosphamide. *Transplantation.* **2**:475.
26. Sutton, W. T., F. van Hagen, B. H. Griffith, and F. W. Preston. 1963. Drug effects on survival of homografts of skins. *Arch. Surg.* **87**:840.
27. Santos, G. W., and A. H. Owens, Jr. 1965. A comparison of the effects of selected cytotoxic agents on allogeneic skin graft survival in rats. *Bull. Johns Hopkins Hosp.* **116**:327.
28. Humphreys, S. R., J. P. Glynn, and A. Goldin. 1963. Suppression of the homograft response by pretreatment with antitumor agents. *Transplantation.* **1**:65.
29. Santos, G. W., and A. H. Owens, Jr. 1964. A comparison of the effects of selected cytotoxic agents on the primary agglutinin response in rats injected with sheep erythrocytes. *Bull. Johns Hopkins Hosp.* **114**:384.
30. Uy, Q. L., T. Srinivasan, G. W. Santos, and A. H. Owens, Jr. 1966. Effects of selected cytotoxic agents on the primary immune response in mice. *Exptl. Hematol.* **10**:4.
31. Cole, L. J., and M. E. Ellis. 1958. Delayed deaths in sublethally x-rayed F_1 hybrid mice injected with parental strain spleen cells. *Science.* **128**:32.
32. Kaplan, H. S., and B. H. Rosston. 1959. Studies on a wasting disease induced in F_1 hybrid mice injected with parental strain lymphoid cells. *Stanford Med. Bull.* **17**:77.
33. Cudkowicz, G. 1965. The immunogenetic basis of hybrid resistance to parental marrow grafts. *In* Isoantigens and Cell Interactions. J. Palm, editor. The Wistar Institute Press, Philadelphia.
34. Glynn, J. P., A. Fefer, and B. L. Halpern. 1968. Cyclophosphamide-induced chimerism. *Cancer. Res.* **28**:41.
35. Billingham, R. E., and W. K. Silvers. 1961. Quantitative studies on the ability of cells of different origins to induce tolerance of skin homografts and cause runt disease in neonatal mice. *J. Exptl. Zool.* **148**:113.
36. Santos, G. W., and A. H. Owens, Jr. 1967. Production of wasting disease and lymphoid chimerism in mice with cylophosphamide and allogeneic marrow. *Federation Proc.* **26**:639.
37. Santos, G. W., and A. H. Owens, Jr. 1968. Syngeneic and allogeneic marrow transplants in the cyclophosphamide pretreated rat. *In* Advance in Transplantation. Proceedings of the First International Congress of the Transplantation Society, Paris, 27–30 June 1967. J. Dausset, J. Hamburger, and G. Mathe, editors. Munksgaard, Copenhagen. 431.