Allogeneic Marrow Engraftment by Cross Circulation in Lethally Irradiated Dogs

By R. B. Epstein, T. C. Graham, C. D. Buckner, J. Bryant and E. D. Thomas

Allogeneic bone marrow transplantation has been accomplished in a number of animal species, including man. Major problems in successful attainment of such allogeneic marrow transplants are (1) sustaining the host through the period of marrow aplasia produced by irradiation or drugs until engraftment has occurred, (2) obtaining and infusing sufficient viable donor marrow elements so that prompt engraftment can occur, and (3) providing a means by which the frequently lethal graft-versus-host reaction can be ameliorated when genetically divergent animals are used for donor-recipient pairs. Current methods of meeting these problems involve the infusion of fresh whole blood and platelet preparations during the aplastic period, the use of multiple marrow donors to provide greater numbers of precursor marrow elements from which the host may select the most compatible donor tissue, and therapy with immunosuppressive agents to enhance graft acceptance and ameliorate the severe forms of homologous disease occurring in out-bred populations. These methods, however, have met with only limited success in large animals. New technics for obtaining successful marrow grafts and supporting the irradiated animal during the period of aplasia are desirable.

Studies in mice, guinea pigs and dogs have demonstrated that cells capable of marrow repopulation are present in the circulating blood. Evidence that such a circulating stem cell population exists in normal human beings remains in doubt. Following lethal irradiation in dogs, marrow repopulation has been achieved with infusion of stored peripheral, autologous leukocytes, and an occasional dog may show evidence of marrow repopulation following intusions of homologous fresh blood.

The development of technics of cross circulation in the dog by means of...
indwelling, teflon-silastic shunts has provided a means of testing the efficacy of peripheral blood from a donor with normal marrow function in establishing a marrow graft in a recipient animal rendered aplastic by an otherwise lethal dose of whole-body irradiation. In addition, cross circulation offers a means of effective transfer of platelets and leukocytes in numbers not attainable by other technics for support of the animal with marrow aplasia. The present studies were undertaken in the dog to investigate the feasibility and the natural history of allogeneic marrow engraftment by this technic.

**Materials and Methods**

Unrelated beagle and mongrel dogs, weighing 8 to 20 kilograms, were matched for canine red cell antigen A, immunized against hepatitis and distemper, dewormed and observed for a period of 3 weeks before use. Radiated animals received 1200 r of total-body irradiation delivered by two opposing cobalt sources at a distance of 2 meters and at a dose rate of 9.2 r per minute. The radiation dose was determined with a Victoreen “r” meter and confirmed by thermoluminescent dosimetry.

On the day following irradiation, under pentobarbital anesthesia, teflon-silastic shunts were placed in the carotid artery and jugular vein of the irradiated animal and his normal partner. Cross circulation was initiated by connecting the arterial cannula of each dog with the venous cannula of his partner by a 24-inch piece of silastic tubing (internal diameter of 2.6 mm.). At the termination of the procedure the dogs were separated and the arteriovenous cannulas were joined with a small teflon connector (see Fig. 1). The external shunt was stabilized by taping it to a formed metal support and the neck was wrapped with gauze and a large cloth collar. Cross circulation for 2 to 6 hours was carried out daily (with a few exceptions) for 6 to 9 days. At the termination of the cross circulation program, the shunts were tied off and removed. The dogs were supported with fluid replacement during periods of vomiting or diarrhea. Antibiotics were used to treat clinical infection. Autopsies were performed upon the death of the experimental animal, and one dog, 105, was sacrificed in a moribund state.

Three groups of dogs were studied: Group I. 5 control dogs that received 1200 r and no further therapy except antibiotics and fluids as indicated by their clinical course; Group II, 5 dogs that received 1200 r. cross circulation and methotrexate (.25 mg./Kg. on days 1. 3 and 5 and 125 mg./Kg. on subsequent days depending on clinical evidence of toxicity). White blood cell counts, platelet counts and hematocrits were performed prior to the cross circulation each day. Evidence of bone marrow repopulation was assessed by a spontaneous rise in formed blood elements after cross circulation was discontinued and by bone marrow histology.

The presence of female drumstick markers in male recipients of female donors was determined in those animals whose white blood cell count rose to sufficient levels for accurate determination. In one pair of dogs cytogenetic studies were carried out to determine the sex karyotype of mitotable cells derived from the bone marrow and peripheral blood. Chromosomal analysis was carried out by a modification of the short-term culture technic of Moorehead et al. Direct bone marrow preparations were also made.

**Results**

*Technical Considerations.* By our modification of the Quinton-Dillard-Scribner shunt, cross circulation could be carried out without anticoagulation in the unanesthetized animal. A pump was not required, as arterial flows through the shunt averaged approximately 200 ml. per minute. The system was self-regulating and imbalance of flow rarely occurred. As a precautionary measure, one of the animals was usually placed on a sensitive scale which permitted early detection of significant imbalances of flow. Most shunts remained in
place throughout the period of study and were tied off following the last cross circulation. In instances where clotting of the shunt occurred before the cross circulation program was complete, a second shunt was placed in the contralateral carotid and jugular vessels. It was found that a teflon felt collar, buried in the subcutaneous tissue during placement of the shunt, provided a matrix for tissue ingrowth and gave both a measure of stability and resistance to infection at the shunt site. Infection in the area of the shunt was not a problem in the present studies and all wounds healed well following the removal of the shunt. This is of particular importance in dogs receiving irradiation, since operative procedures in such animals are frequently complicated by poor wound healing and severe infections.

Survival. Group I, animals receiving 1200 r, survived between 4 and 11 days with a mean of 7.2 days. Group II, animals that were only cross circulated, survived between 7 and 12 days with a mean of 8.8 days. Group III, animals receiving methotrexate and cross circulation, survived between 17 and 21 days with a mean of 19.8 days (Tables 1 and 2).
Evidence of Marrow Repopulation. Histologic examination of bone marrow preparations of control animals revealed total absence of myeloid, erythroid and megakaryocytic elements. Tables 1 and 2 summarize the treatment schedules, general hematologic findings and bone marrow histology in Groups II and III. Figures 2 and 3 show the white blood cell counts and platelet counts in the three groups. Figures 4A, B and C show details of treatment schedules and changes in white cells and platelets in representative animals of the three groups. In Group II, animals not receiving methotrexate, a rise in white blood cell count was noted in 3 of the 5 animals, but none had a significant rise in platelet count. At autopsy, however, erythroid and myeloid precursors were seen in the marrows of all animals (Fig. 5).

In Group III, those treated with methotrexate, there was clear peripheral blood evidence of repopulation in all instances. This was manifested by a rise in white blood cell count in each of the 5 animals and a more delayed rise in platelet count in 4 of the 5 animals. All of these dogs had a cellular marrow at postmortem examination (Fig. 5).
Table 1.—Dogs Treated with 1200 r and Cross Circulation

| Dog # | Cross Metho- No. Kg./ Rise in Rise in Bone Marrow | Circulation trate Sur- Platelets Marrow | Day Hrs. vival in Days | WBC | History |
|-------|--------------------------------|-------------------------------------|-----------------|-----|---------|-------------|
| 281   | 1 4 0 12 Yes (to 3600) | None                              | Moderate cellularity |
| 93    | 1 4 0 10 Yes (to 1800) | None                              | Minimal cellularity, myeloid and erythroid elements present |
| 103   | 1 4 0 8 None (860 at death) | None                              | Moderate cellularity |
| 575   | 1 4.7 2 0 7 Yes (to 2200) | None                              | Minimal cellularity, myeloid and erythroid elements present |
| 92    | 1 4 0 7 None (1300 at death) | None                              | Poor cellularity, few myeloid and erythroid elements present |

In pairs of animals in which female donors were used as cross circulation partners with male recipients, drumstick counts of mature peripheral neutrophils were obtained shortly before death and from 10 days to 2 weeks after the last cross circulation. In 3 of the 4 male recipients studied, drumsticks ranged from 2 to 6 per cent and corresponded to the count in the respective female partner.

Figure 6A shows the chromosomes arranged in karyotype from a leukocyte culture of a male dog prior to 1200 r of irradiation. Figure 6B shows the typical female karyotype prepared from direct bone marrow smear of this male dog 20 days after irradiation and 2 weeks following the last cross circulation. Table 3 shows the results obtained from analysis of the direct marrow and cultured blood preparations. Among 75 cells counted on direct marrow smear, 50 contained the full complement of 78 chromosomes and all had XX chromosomes. Of 54 cells counted from short-term peripheral blood cultures, 50 were complete with 78 chromosomes, and only female donor cells were found. Thus, all cells observed in this male dog 3 weeks after irradiation and 2 weeks after the last period of cross circulation were of female donor type.

Evidence of Homologous Disease. These animals with allogeneic marrow repopulation all died with clinical and histologic findings consistent with what has been described in forms of secondary disease seen in other animal species. Clinically, these included diarrhea, weight loss, dermatitis, jaundice and susceptibility to infection. Pathologically, hepatocellular degeneration, cryptic degenerative changes in the gut, depletion of follicular nodal structures, and widespread presence in tissues of immature mononuclear forms staining heavily with RNA stains were consistently observed. Representative histologic findings are shown in Figure 7.
Fig. 2.—White blood cells per cu. mm. in the three groups of dogs. During the period of cross circulation, blood for counts was obtained just before each day’s cross circulation.

Fig. 3.—Platelets per cu. mm. in the three groups of dogs. During the period of cross circulation, blood for counts was obtained just before each day’s cross circulation.
<table>
<thead>
<tr>
<th>Dog #</th>
<th>Cross Circulation Methotrexate</th>
<th>Methotrexate</th>
<th>Survival in Days</th>
<th>Rise in WBC</th>
<th>Rise in Platelets</th>
<th>Bone Marrow Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>1</td>
<td>1.35</td>
<td>25</td>
<td>Yes (to 10,000)</td>
<td>Yes to (150,000)</td>
<td>Cellular</td>
</tr>
<tr>
<td>81</td>
<td>2</td>
<td>1.35</td>
<td>8</td>
<td>Yes (to 3,300)</td>
<td>Yes to (10,000)</td>
<td>Cellular</td>
</tr>
<tr>
<td>58</td>
<td>3</td>
<td>1.35</td>
<td>25</td>
<td>Yes (to 7,000)</td>
<td>Yes to (4,000)</td>
<td>None</td>
</tr>
<tr>
<td>77</td>
<td>4</td>
<td>1.35</td>
<td>25</td>
<td>Yes (to 10,000)</td>
<td>Yes to (25,000)</td>
<td>None</td>
</tr>
<tr>
<td>105</td>
<td>3</td>
<td>1.35</td>
<td>3,4</td>
<td>Yes (to 8,000)</td>
<td>Yes to (6,000)</td>
<td>Cellular</td>
</tr>
</tbody>
</table>
Fig. 4A.—Leukocyte and platelet counts of a control dog following 1200 r of whole-body irradiation.

Fig. 4B.—Leukocyte and platelet counts and cross circulation schedule of dog 281.
Fig. 4C.—Leukocyte and platelet counts and cross circulation and methotrexate schedule of dog 60.

DISCUSSION

The results reported here show clearly that marrow allografts in lethally irradiated dogs can be produced with peripheral blood from a normal donor dog by cross circulation. Marrow engraftment occurs within 2 weeks, as shown by marrow histology and the appearance of formed blood elements in increasing numbers. Evidence that allogeneic engraftment has occurred, rather than autogenous regeneration, rests on the prompt appearance of marrow repopulation in the face of irradiation in the 1200 r range, the subsequent development of a lethal secondary syndrome with prolongation of survival by methotrexate, the presence of female drumsticks on granulocytes of male recipients, and the cytogenetic demonstration of donor cells in marrow and peripheral blood of the cross circulation recipient. Such evidence, although not excluding participation of humoral factors, clearly indicates repopulation by donor cells.

These findings are in agreement with other reports indicating that cells capable of marrow repopulation are present in the peripheral blood. Work by Cavins et al. in the dog has shown that preparations of stored autologous peripheral white cells can serve as a source of cells for marrow repopulation. In contrast to the findings in rodents and dogs, Kurnick et al. have presented evidence that, in man, areas of marrow hypoplasia produced by local irradiation remain hypoplastic unless autologous marrow is infused, suggesting an absence of a circulating stem cell population. Temporary chimerism, however, has apparently been produced in patients with marrow
Fig. 5.—(a) Marrow aplasia seen in a control dog 10 days following 1200 r of whole-body irradiation. (H.E.)
(b) Early marrow repopulation 10 days after 1200 r of whole-body irradiation in a cross-circulated dog. H.E., 220 x.
(c) Cellular marrow 21 days after 1200 r of whole-body irradiation in a dog cross-circulated and given methotrexate. (H.E.)
(d) Area of erythroid recovery in a cross-circulated dog. (Wright's)
(e) Area of granulocytic regeneration in a methotrexate cross-circulated dog. (Wright's)
(f) Megakaryocyte in a methotrexate-treated, cross-circulated dog. (Wright's)
Fig. 6A—Canine male karyotype prior to 1200 r.

Fig. 6B.—Canine female karyotype prepared from bone marrow of a male dog cross-circulated with a female partner.
hypoplasia secondary to antileukemic therapy who have been treated with infusions of peripheral white blood cells from donors with chronic myelogenous leukemia. The identification of the circulating cells responsible for the marrow repopulation noted in the present studies remains obscure, although the limited number of morphologic types in peripheral blood leukocytes as opposed to the heterogeneity of bone marrow cells could perhaps offer an approach for further qualitative and quantitative studies of the stem cell population. The accessibility of peripheral blood elements may also offer an alternative or supplementary source for greater quantities of cells with marrow repopulation potential than are currently available from single marrow donors in man or large animals.

The silastic-teflon shunts are described in detail because of the advantages of maintaining such a shunt in the dog for long periods of time without anticoagulation or anesthesia. Such a system offers the unique advantage of supplying daily infusions of large numbers of fresh granulocytes and platelets to the animal with marrow aplasia during the lag period before effective engraftment occurs, a period in which death from infection or hemorrhage is most apt to occur.

The consistent severity of the secondary disease noted in these dogs contrasts with the variable course noted in dog populations receiving allogeneic marrow. These findings resemble the severe, early, secondary disease syndromes noted in homologous mouse systems when peripheral blood or lymph node cells are added to donor marrow. The daily infusion of immunologically active donor cells via cross circulation probably accounts for the early, severe secondary disease syndrome noted. The methotrexate-treated group survived significantly longer than dogs treated with cross circulation alone. This finding is consistent with the observation that methotrexate is a potent immunosuppressive agent and is capable of ameliorating the so-called graft-versus-host reaction. The particular regimen of cross circulation and methotrexate treatment used in the present studies was arbitrarily selected and other schedules of cross circulation and drug administration remain to be investigated. The uniformity of survival and clinical and histologic findings makes these dogs a convenient model for testing regimens designed to ameliorate this reaction.

Pathologic findings in these animals are in agreement with those previously reported in allogeneic marrow graft studies. Tissue changes include hepatic necrosis, cryptic degenerative changes in the gastrointestinal tract, and loss of follicular nodal structure. In this series of dogs clinical jaundice with post-

### Table 3.—Chromosomes of XY Dog Treated with XX Cells (20 Days Postirradiation)

<table>
<thead>
<tr>
<th>Chromosome No:</th>
<th>&lt;78</th>
<th>78</th>
<th>&gt;78</th>
<th>XX</th>
<th>XY</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marrow</td>
<td>25</td>
<td>50</td>
<td>0</td>
<td>75</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>3</td>
<td>50</td>
<td>1</td>
<td>54</td>
<td>0</td>
<td>54</td>
</tr>
</tbody>
</table>

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Fig. 7.—Histologic changes noted approximately 3 weeks following lethal irradiation in cross-circulated animal's dying with homologous marrow grafts.
(a) Lymph node: lymphoid depletion and absence of follicular structure. (H.E.)
(b) Liver: hepatocellular degeneration. (H.E.)
(c) Skin: dyskeratotic changes. (H.E.)
(d) Ileum: cryptic degenerative changes. (H.E.)
(e) Ileum: mononuclear infiltration of the submucosa. (H.E.)
(f) Ileum: mononuclear infiltration of the submucosa. (H.E.)
mortem evidence of severe hepatocellular degeneration was frequently observed. These findings were also noted by Collins in several supralethally irradiated dogs thought to have marrow grafts following multiple blood transfusions. Severed hepatocellular changes were also described in monkeys dying of secondary disease following homologous marrow infusions. Although the pathologic findings are difficult to interpret in the face of radiation and possible terminal infectious processes, dogs given autologous marrow go on to complete and uncomplicated recovery.

The cytogenetic identification of cells from the bone marrow and peripheral blood proved to be a relatively simple procedure when female donors were used for male recipients. The prominent XX chromosomes of the female make a useful tool for identification of dividing cell populations in the dog.

**Summary**

Dogs given 1200 r of total body irradiation were cross-circulated with dogs having normal marrow function. Irradiated controls died in from 4 to 11 days with marrow aplasia. Dogs cross-circulated daily for 6 to 9 days showed histologic evidence of bone marrow repopulation after 1 week. Male dogs cross-circulated with female partners showed typical female drumsticks on mature granulocytes after repopulation had occurred. Cytogenetic studies of an irradiated male dog cross-circulated with a female partner showed all mitotable cells from the bone marrow and peripheral blood to be of female donor type. Allogeneic bone marrow engraftment was associated with an early and severe secondy syndrome which resulted in the death of the animals in the second week. When methotrexate was given, survival was increased to 3 weeks.

It was concluded that (1) cross circulation provided leukocytes and platlets adequate for support during the period of radiation-induced marrow aplasia, (2) allogeneic marrow engraftment was produced consistently by cells transferred in the peripheral blood of the normal cross circulation partner, (3) the grafts were associated with an early and severe form of secondary disease, and (4) methotrexate given during the early period of engraftment reduced the severity of the secondary disease.

**Summario in Interlingua**

Canes tractate con 1200 r de irradiation del corpore total esseva coniectite in circulation cruciate con canes in que le functionamento del medulla esseva normal. Irradiate animales de controlo moriva in inter 4 e 11 dies in consequentia de aplasia medullari. Canes subjeite al circulation cruciato omne die durante inter 6 e 9 dies manifestava evidentia histologica de un repopulation del medulla ossee post un intervallo de un septimana. Canes mascule in circulation cruciato con partenarios feminin monstrava tipicamente feminin bastones de tambor in lor granulocytos matur post que le repopulation haveva occurrit. Studios cytogenetic in un irradiate can mascule in circulation cruciato con un partenario feminin monstrava que omne le cellulas capace de mitose in le medulla ossee e in le sanguine peripheric esseva del typo feminin del donator. Graffage de medulla ossee allothigenic esseva associate con un
precoce e sever syndrome secundari resultante in le morte del animales durante le secunde septimana. Post le administration de methotrexato, le superviventa esseva prolongate a un total de 3 septimanas.

Es conclutite que (1) le circulation cruciate provideva leucocytos e plachet-tas in quantitates adequate pro supportar le vita del animales durante le periodo de aplasia medullari inducite per irradiation, (2) graffage de medulla allothigenic esseva producite uniformemente per celular transferite ad in le sanguine peripheric del partenario normal in le circulation cruciate, (3) le graffos esseva associate con un precoce e sever forma de morbidity secundari, e (4) methotrexato administrate durante le phases initial del graffage reduceva le severitate del morbo secundari.

ACKNOWLEDGMENTS

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REFERENCES

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ALLOGENEIC MARROW ENGRAFTMENT BY CROSS CIRCULATION


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