Mixed Leukocyte Reactions and Histocompatibility in Rats

Abstract. *Mixed leukocyte culture tests have been carried out on two heterogeneous but genetically defined backcross populations of rats to determine whether the reactions observed can provide the basis for histocompatibility matching. The experiments were designed so that only one-way reactions could occur. Only when the donors of individual leukocyte mixtures differed at the important* Ag-B *histocompatibility locus was there any in vitro reactivity, and differences at this locus were invariably associated with the prompt rejection of skin homografts. Determination of compatibility at this locus proved to be important in that it facilitated the prolongation of survival of skin homografts by immunosuppressive therapy.*

When blood leukocytes from two unrelated individuals are mixed and maintained in culture for several days, a mutual proliferative response occurs which is characterized by the emergence of blast cells and increased DNA synthesis (*1*, *2*). This response appears to be causally related to the immunogenetic disparity between the cell donors and is currently under investigation as a means of selecting donors for kidney transplants. The matching procedure entails incubating leukocytes from the propositus with cells from each member of a donor panel and selecting that donor whose cells evoke the smallest reaction.

To date some success has attended the employment of this test to predict the fate of skin homografts in man (*3*) and the results have been successfully correlated with the degree of cross-reactivity of skin grafts from two individuals placed on a third, unrelated recipient (*1*). However, this test is difficult to evaluate in man, as in this species genetic heterogeneity precludes accurate definition of immunogenetic relationships and makes two-way reactions almost inevitable.

To overcome this impasse we have directed our attention to rats, where the availability of isogenic strains makes it possible to establish genetically defined heterogeneous populations in which one-way leukocyte reactions can be assessed and quantitated. A comparison has been made of in vitro leukocyte reactions, skin graft survival time, and compatibility for antigens determined by the important *Ag-B* histocompatibility locus (*4*) in this species. The tests for these three parameters were performed as independent "blind" studies.

Rats of the Lewis (*Ag-B*^{1}), BN (*Ag-B*^{3}), and DA (*Ag-B*^{4}) strains were used. In one study DA/Lewis F_1 hybrid males were backcrossed to Lewis females. The offspring of such matings (R_2) vary with respect to the number of specific DA factors received from the hybrid parent but all inherit a complete set of histocompatibility genes from the Lewis parent. Hence, in theory, any activity that is detected when blood leukocytes from Lewis rats are mixed in vitro with those of a member of the R_2 population must represent a one-way reaction, the reaction of Lewis leukocytes against the foreign DA antigens present on the leukocytes of that particular backcross animal. If the magnitude of this reaction is related to histocompatibility, then it should be of some value in predicting the survival of skin grafts. Accordingly, a comparison was made between the in vitro reaction evoked by leukocytes from various members of the R_2 population mixed with Lewis cells, and the survival times of skin grafts from these animals on Lewis recipients. Another backcross population derived from DA females × BN/DA males was also tested. In this case DA animals served as leukocyte donors and as graft recipients.

All cultures were carried out in triplicate. Each contained a mixture of 1 million peripheral blood lymphocytes from an R_2 donor and the same number of parental strain cells in 1 ml of culture medium with 15 percent fresh rat serum. Tritiated thymidine (0.1 ml containing 0.25 μc; 6.7 c/mmole) was added at various times and the cultures were harvested 16 hours later, on the 4th to 7th day, and washed with saline.

Animal Number	AgB Genotype	Graft Survival Time(days)	MLR Reaction
♀1	1/4	<8	+
♂5	1/4	8	+
♂80	1/1	>50	-
♀6	1/1	29	-
♂3	1/1	21	-
♂6	1/1	8	-
♂2	1/1	11	-

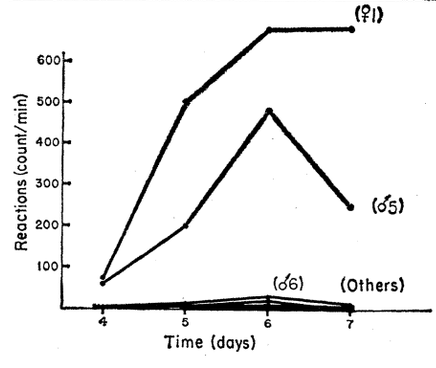

Fig. 1 (right). Mixed leukocyte reactions (MLR) in a typical experiment. The reactions are represented in counts per minute of H^3-thymidine in cultures of Lewis and R_2 cells. Note that positive reactions are correlated with *Ag-B* genotype and not with graft survival time.

Table 1. The survival of skin grafts from backcross rats on *Ag-B* compatible and incompatible recipients.

Donors	Donors' *Ag-B* genotype	Host and *Ag-B* genotype	Survival (days)
DA/Lewis × Lewis	1/1	Lewis (1/1)	8, 11, 12, 12, 14, 15, 21, 29, > 100
DA/Lewis × Lewis	1/4	Lewis (1/1)	≤ 8 (18 grafts), 9, 10
BN/DA × DA	4/4	DA (4/4)	10, 10, 12, 12, 13, 14, 14, 14, 14, 16, 19, 26, > 100
BN/DA × DA	3/4	DA (4/4)	≤ 8 (11 grafts), 9, 9, 9, 9, 10, 10, 12

Table 2. Effect of cyclophosphamide in prolonging the survival of *Ag-B* compatible and incompatible grafts.

Treatment	Survival of grafts (days)	
	Ag-B incompatible	*Ag-B* compatible
Single inoculation of 7.5 mg/100 g	8, 9, 9, 9, 10, 10,	9, 10, 10, 12, 14, 18
Three inoculations of 2 mg/100 g	9, 11, 11, 11, 12, 12	13, 14, 14, 15, 16*, 18
No inoculation	8, 8, 8, 9	8, 8, 9, 9

* Animal died.

An acid-insoluble (trichloroacetic acid) precipitate was prepared and collected onto glass-fiber filter pads, and the radioactivity was assessed with liquid scintillation counting techniques (*5*). The *Ag-B* genotypes of the backcross progeny were identified by red cell hemagglutination with specific isoimmune reagents (*4*). Test skin grafts were prepared from the ears of each R_2 member and were transplanted to an appropriate host (*6*). Grafts were scored daily following the initial 8-day inspection.

The results of the tests conducted with animals of the two R_2 populations were as follows: (i) Significant incorporation of radioactive thymidine was always observed in the leukocyte cultures when the R_2 and the parental strain donors were incompatible at the *Ag-B* locus; when skin was grafted from these particular R_2 donors to the appropriate parental strain, host rejection was complete within 12 days (Table 1). (ii) No appreciable in vitro reactions were incited if the R_2 and parental strain donors of the leukocyte mixtures were alike at the *Ag-B* locus. Skin grafts from backcross donors compatible at this locus survived for varying periods of time (Table 1). Of particular interest was the lack of reactivity in cultures of 8 of 22 animals of this group whose skin grafts were destroyed by the 13th day (Table 1). This is illustrated in Fig. 1, a typical experiment, where the *Ag-B* compatible leukocytes of male No. 6 failed to stimulate those of Lewis origin, even though skin from this animal was rejected by a Lewis host as promptly as skin from male No. 5 and female No. 1, two *Ag-B* incompatible animals whose leukocytes did induce a marked in vitro response.

The results of these experiments indicate that the proliferative response occuring in mixed leukocyte cultures is highly selective in distinguishing between two classes of donor-host combinations which exhibit acute homograft reactions. One results from antigenic differences determined by a major histocompatibility locus—the *Ag-B*—and the other stems from a multiplicity of weaker histocompatibility factors which probably act synergistically (*7*).

These results raised the question of whether immunosuppressive procedures might prolong to different extents the survival of grafts derived from these two groups of donors on parental strain recipients. To provide an answer, three panels of Lewis rats were challenged with two skin grafts, one from an *Ag-B* incompatible and one from an *Ag-B* compatible member of the Lewis/DA × Lewis backcross population whose skin had previously been rejected by Lewis animals by the 8th day. One of these panels of bilaterally grafted animals received a single intraperitoneal inoculation of cyclophosphamide (7.5 mg/100 g) 4 days after grafting, another was inoculated with the drug at 4, 6, and 8 days (2 mg/100 g), and a third was untreated.

The results (Table 2) indicated that a difference in the two groups did exist; the grafts from the *Ag-B* compatible donor fared better than did those from the *Ag-B* incompatible donor when grafted simultaneously on the same drug-treated recipients. The fact that this difference was not more pronounced is not surprising, since the lives of skin homografts, in contrast to those of kidney, are known to be notoriously difficult to prolong with immunosuppressive measures (*8*). For this reason an even greater disparity might be expected if kidneys, rather than skin, were used.

Taken together, these results suggest that although the mixed leukocyte reaction may not be able to detect all levels of histoincompatibility, it may be useful in excluding those combinations which are least amenable to immunosuppressive therapy. In this regard it should be noted that in man there is evidence that a single locus, with multiple alleles, is also responsible for mixed leukocyte reactions (*9*).

WILLYS K. SILVERS
DARCY B. WILSON
Department of Medical Genetics, University of Pennsylvania School of Medicine, Philadelphia
JOY PALM
Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania

References and Notes

1. F. H. Bach and K. Hirschhorn, *Science* **143**, 813 (1964).
2. B. Bain and L. Lowenstein, *ibid.* **145**, 1315 (1964).
3. ——— and L. D. MacLean, in *Histocompatibility Testing* (Publ. 1229, NAS-NRC, Washington, 1965), pp. 121–129; R. Ceppellini *et al.*, in *Histocompatibility Testing 1965* (Munksgaard, Copenhagen, 1965), pp. 13–23.
4. J. Palm, *Transplantation* **2**, 603 (1964).
5. A report containing details of this culture system is in preparation.
6. R. E. Billingham, in *Transplantation of Tissues and Cells*, R. E. Billingham and W. K. Silvers, Eds. (Wistar Institute Press, Philadelphia, 1961), pp. 1–26.
7. R. J. Graff *et al.*, *Transplantation* **4**, 605 (1966).
8. R. V. Moseley *et al.*, *ibid.*, p. 678.
9. F. H. Bach and N. K. Voynow, *Science* **153**, 545 (1966).
10. Supported in part by PHS grants AI-07001 and CA-04534. W.K.S. is a PHS Career Development awardee (K-3-CA-4224) and D.B.W. is a fellow of the Helen Hay Whitney Foundation. The technical assistance of Mrs. Heather Pullen, Mrs. Jane Rogers, and Mrs. Gustava Black is gratefully acknowledged. We are also indebted to R. E. Billingham for helpful criticism and advice.

22 December 1966

Microincision of Sickled Erythrocytes by a Laser Beam

Abstract. *Increased mechanical fragility of the sickled red cell is thought to be important in the genesis of the hemolytic process in sickle-cell disease. Sickled cells were observed cinematographically after microincision by a ruby-laser beam. Distortion and charring invariably occurred at the site of injury, and with small injuries there was no further cell change. With larger injuries, variably rapid retraction of spicules occurred accompanied by sphering of the cell. In some cases, progressive loss of hemoglobin accompanied and followed the changes in shape; in others the sphered cell still contained hemoglobin. Regardless of the mechanisms involved in these changes in vitro, the observations may be applicable to destruction of sickled cells in vivo. We suggest that the cells are subject to avulsion of rigid cellular processes as a result of mechanical injury incurred in normal circulation. Such injured cells may undergo either immediate hemolysis or transformation into spherocytes which are subject to erythophagocytosis.*

Erythrocytic sickling in sickle-cell disease, caused by intracellular polymerization of deoxygenated sickle hemoglobin (*1*), is considered responsible for the major features of the disease, vascular stasis, occlusion and thrombosis, and hemolytic anemia (*2–4*). Our experiments are concerned with the relationship of physical damage of the sickled cell to hemolysis. We have directly observed single sickled cells after discrete injury by a laser beam.

Erythrocytes suspended in plasma from patients with sickle-cell anemia were prepared with and without sodium metabisulfite. Microscopic fields