Induction of Tolerance in Adult Mice with Subcellular Spleen Fractions.* (29094)

R. J. HOWARD, J. M. GORDON, B. POLLARA, C. MARTINEZ, AND R. A. GOOD
Pediatric Research Laboratories, Variety Club Heart Hospital and Department of Physiology, University of Minnesota

Immunologic tolerance of skin grafts may be produced in adult mice by several methods, including injection of viable lymphoreticular cells from the prospective donor strain (1-8). The number of cells required to produce tolerance is dependent upon the degree of histocompatibility difference between donor and host. Thus, in the case of the relatively weak histocompatibility barrier conditioned by the male Y chromosome in C57BL and A strain mice(9), an injection of only 20-40 million viable male spleen cells into the adult female regularly produces tolerance of male skin grafts(1,5). However, when other non-H-2 barriers and to a greater extent when the strong H-2 histocompatibility barrier must be overcome, many more spleen cells are required to produce tolerance during adult life. For example, induction of tolerance in adult C3H/Bi mice to $(A \times C3H)F_1$ skin grafts in one experiment required injection of 1.5×10^8 $(A \times C3H)F_1$ spleen cells over a 7-week period(6).

Recently it has been reported that immunologic tolerance can be produced in both neonatal and adult mice using non-viable cells and cell extracts(10-13). Linder(11) observed that tolerance of $(DBA \times C57BL)F_1$ male skin can be induced in adult female mice of the same strain by repeated injections of cell-free homogenates of male liver, spleen, and kidney. Martinez *et al*(12,13) have achieved tolerance across the weak sex-linked histocompatibility barrier in C57BL mice, across other non-H-2 barriers, and even across the formidable H-2 barrier in adult mice by repeated injections of disrupted spleen cell preparations containing no demonstrable viable cells. Kelly *et al*(14) have found that the tolerance-producing material can be obtained from disrupted cell preparations of liver and kidney as well as spleen.

This report concerns an extension of these investigations. In this study subfractions of disrupted spleen cells have been evaluated for their capacity to produce tolerance in adult female C57BL mice of syngeneic male skin. We have found that the subcellular particulate fraction and specifically the microsomal fraction contain the tolerance producing material and, indeed, may be as effective in producing a tolerant state across the sex-linked transplantation barrier as are whole spleen cells.

Materials and methods. Male C57BL/1 mice, 45 to 180 days of age, were sacrificed, their spleens removed and prepared as cell-free homogenates(13). Three subcellular fractions were prepared: the *particulate fraction,* sediment from centrifugation of the homogenate at 15,000 *g* (average) for 10 minutes, containing cell walls, nuclei, mitochondria, and other large cellular debris; the *microsome fraction,* sedimented by centrifugation of the supernate at 105,000 *g* (average) for 60 minutes; and the *soluble fraction,* the final supernate. The particulate and microsome fractions were each washed in Ringer's lactate solution and recentrifuged, followed by suspension in Ringer's lactate solution, 2 spleen equivalents per ml.

A *"Tris" extracted particulate fraction* was prepared by extraction of the spleen homogenate for 30 minutes with 0.1 M, pH 8.7, trihydroxy-methyl-amino-methane buffer ("Tris"), followed by centrifugation at 15,000 *g* (average) for 20 minutes and resuspension in Ringer's lactate solution, 2 spleen equivalents per ml.

Preparation of an *RNASE-treated microsome fraction* involved suspension of the microsome fraction in normal saline, 4 spleen equivalents per ml, and incubation with 0.05 mg per ml of bovine pancreatic RNASE (Sigma Chemical Co.), 1.0 mg/ml "Tris"

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TABLE I. Acceptance of Syngeneic Male Skin Grafts by Adult Female C57BL/1 Mice After Repeated Injections of Subcellular Fractions.

Cell fraction	No. of injections*	Rate of graft acceptance† No.	%
Particulate fraction	12	5/7	71
Microsome "	12	6/8	75
Soluble "	12	2/8	25
RNASE treated microsome fraction	11	6/11	55
"Tris" extracted particulate fraction	11	9/11	81
"Tris" buffer extract controls	11	0/12	0
RNASE controls	11	0/10	0
Uninjected "	0	2/25	8

* Each injection included the equivalent of 2 spleens.

† Survival of grafts considered accepted was verified at 2 mo and at 5½ mo.

acetate buffer, and 0.005 M EDTA (ethylene-diamine-tetraacetic acid), pH 7.5, for 1 hour at 20°C, followed by exhaustive dialysis at 4°C against 0.01 M "Tris" acetate buffer in 0.005 M EDTA for 24 hours.

Each fraction was diluted to 2 spleen equivalents, and administered twice a week, first by the intravenous route and intraperitoneally thereafter to female recipients, 45 to 75 days old. They were grafted at the time of the third injection with skin from male donors, 45 to 75 days of age, by the technique routinely used in our laboratory (15). Semiweekly injections were continued for 4-5 additional weeks (total of 11 or 12 injections). Rate of graft survival was determined 2 months after placement.

Control animals received RNASE solution in 0.01 M "Tris" acetate buffer, "Tris" buffer alone, or no treatment at all.

Results. The results are summarized in Table I. It is apparent that both the microsomal and particulate fractions are active in producing immunologic tolerance in this system. Five of 7 female mice injected with the particulate fraction accepted syngeneic male skin as did 6 of 8 females injected with the microsomal fraction. However, only 2 of 8 injected with the soluble fraction were tolerant. Of the untreated control females, 2 of 25 accepted the syngeneic male skin grafts.

It was further found (Table I) that repeated "Tris" buffer extractions of the particulate fraction and RNASE digestion of the microsomal fraction did not eliminate the capacity of these subcellular fractions to induce a tolerant state. Female mice given "Tris" buffer alone or RNASE alone in concentrations similar to those contained in the cell fractions did not accept male skin grafts.

Discussion. These observations are of interest from several points of view. First, they demonstrate again that in a system involving a relatively weak histocompatibility barrier a tolerant state can regularly be produced during adult life by repeated injections of subcellular materials. Separation based upon centrifugation indicates that the cell fractions involved include the relatively crude "particulate" fraction containing mitochondria, cell walls and nuclei, and the microsomal fraction. Little or no activity was present in the soluble supernatant material. Repeated extraction with "Tris" buffer to remove soluble protein and digestion with RNASE did not reduce significantly the capacity to produce a tolerant state in this system.

Evidence from several quarters based upon sensitization with partially characterized cellular subfractions(16,17) suggests that some transplantation antigens are associated with membrane lipoproteins. In Manson's(18) studies it was of interest that no sensitizing material could be demonstrated in preparations made from liver cells. In the studies reported here and in other studies using this system, we have seen no evidence of sensitization with the subcellular preparations under study. It is of interest, however, that Kelly *et al*(14) found liver cells, initially disrupted as were the spleen cells used in this study and in other studies from these laboratories, to be an excellent source of material capable of producing specific immunologic tolerance.

The results of these studies would be consistent with conclusions from prior experiments, namely, that tolerance across a variety of histocompatibility barriers can be produced during adult life. Conclusions as to the exact nature of the tolerance producing

material cannot be made at this time except to state that the material appears to be particulate in the general sense and is probably not intact RNA available to digestion with pancreatic RNASE.

The findings emphasize again that tolerance of allogeneic skin is probably not a unique phenomenon, but is essentially the same as immunologic paralysis and protein antigen overloading.

Summary. Subcellular fractions of C57BL/1 adult male mouse spleen were prepared by homogenization and centrifugation, and administered to prospective skin graft recipients, adult female C57BL/1 animals. Two fractions induced tolerance in a majority of the animals: a particulate fraction and a microsome fraction. Neither "Tris" extraction of the particulate fraction nor RNASE treatment of the microsome fraction affected the rate of tolerance induction significantly.

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Genetic Aspects of Salivary Secretion of Isoagglutinins.* (29095)

ROBERT M. WILSON AND GORDON E. GREEN
Division of Dentistry, Henry Ford Hospital, Detroit, Mich.

In a previous study where an association between immunity to human dental caries and blood group characteristics was sought, an unusually large number of salivary isoagglutinin secretors were found among caries-immune individuals(1). That prompted this study of the probability of genetic control over the secretion of salivary isoagglutinins. Other investigators(2-7) have observed isoagglutinins in human saliva. Furuhata *et al* (4) have postulated genetic control by a pair of recessive genes, however Prokop(5, 6) found salivary isoagglutinins much more frequently in blood type O individuals and rejected Furuhata's hypothesis.

Materials and methods. Two hundred and one humans were used in this study, of whom 60 were selected from families in which immunity was not evident, 81 were related distantly to a caries-immune (first cousin or more distant) and a group of 60 caries-immunes and their close blood relatives. This last group was used only in mating studies because of the published association of caries-immunity and isoagglutinin secretion.

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