CELL-MEDIATED IMMUNITY AND BLOCKING SERUM ACTIVITY TO TOLERATED ALLOGRAFTS IN RATS*

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Animals neonatally inoculated with allogeneic tissues often become tolerant, i.e., capable of later in life accepting allografts containing the respective antigens (1). A widely accepted explanation for tolerance induced by neonatal inoculation of allogeneic cells postulates that those lymphoid cell clones that would have been capable of reacting against the tolerated antigens have been either killed or irreversibly suppressed from reacting (2, 3). It has also been hypothesized, however, that at least part of the tolerance phenomenon, as induced against allografts in newborn animals, is due to the appearance of serum factors capable of blocking otherwise reactive lymphocytes from destroying cells carrying the tolerated antigens (4, 5). There are some recent reports supporting this concept. For example, mice made neonatally tolerant against allografts, as well as tetraparental (allophenic) mice, possess both a cellular immunity against the tolerated tissues (detected by in vitro tests for lymphocyte-mediated cytotoxicity) and a blocking serum activity, i.e. an ability of serum from the tolerant animals to specifically abrogate destruction by immune lymphocytes of cells carrying the tolerated antigens (6-9), and enhancement of tumor allografts has been shown after transfer of serum from mice considered to be tolerant in the classical sense (4). Furthermore, the coexistence of cell-mediated immunity and blocking serum activity has been detected in dogs, mice, and human patients, repopulated with foreign bone marrow after X-irradiation (10-12).

It has been pointed out (13, 14) that the concept that animals tolerant to allografts have blocking serum factors and cellular immunity to the tolerated tissues is incompatible with several reported observations: there have been repeated failures to transfer tolerance with serum, the tolerant state can be broken by inoculation of nontolerant syngeneic cells (15-17), lymphocytes from tolerant rats are specifically incapable (as compared with controls) of synthesizing DNA upon contact with the tolerated alloantigens (18, 19), and mice parabiosed with tolerant syngeneic animals do not become tolerant, as demonstrated by skin grafting immediately upon their separation from the tolerant partners (20).

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In order to clarify whether or not the blocking serum activity, detected in vitro, plays any role in the establishment and/or maintenance of tolerance in vivo, there is a need for studies in which the same animals are serially tested for tolerance in vivo and in which the tolerance is correlated with cell-mediated immunity and blocking serum activity in vitro after various manipulations known to induce or to break tolerance. Analogous “vertical” studies on tumor-bearing rats have been helpful in elucidating the role of blocking serum activity for tumor growth in vivo (21). As one step in this direction, we have tried to induce allograft tolerance by neonatally inoculating B/N and W/Fu rats with allogeneic (W/Fu or B/N) bone marrow cells, following previously published procedures (16, 17); rats were referred to as tolerant if they accepted skin grafts from the strain to which tolerance induction had been attempted for at least 50 (generally more than 100) days. The subject of this paper is the examination of cell-mediated immunity and blocking serum activity, as detected in vitro by using a microcytotoxicity test (22), and to study the correlation of the blocking serum activity with the ability of such rats to accept a skin graft from the respective “tolerated” strain.

Materials and Methods

Animals.—Rats of the inbred (brother-sister mated) W/Fu and B/N strains were used. These rats permanently accept skin grafts within each strain. They were maintained on a standard pellet diet and water given ad libitum.

Induction of Tolerance in Newborn Rats.—W/Fu and B/N rats were neonatally inoculated with allogeneic (B/N and W/Fu) bone marrow cells, as described below.

The long bones (humerus, femur, and tibia) and the iliac bones were removed aseptically from adult female rats that were used as donors. Bone marrow cells were flushed out by forcing Eagle’s F12 medium (containing 1 U heparin/ml) through the bone marrow canals. The cell suspensions were filtered through surgical gauze, centrifuged for 15 min at 220 g, and washed twice. Cell viability was checked by trypan blue exclusion.

Newborn rats (6-12 h old) were inoculated through the anterior facial vein with the allogeneic bone marrow cells, using established procedures (16, 17). W/Fu newborn rats received $40-42 \times 10^6$ nucleated cells from B/N rats, suspended in 0.1 ml of F12 medium; whereas, B/N newborns received $18-20 \times 10^6$ W/Fu nucleated cells, also suspended in 0.1 ml vol.

Rats were referred to as tolerant if they accepted skin grafts from the strain to which tolerance induction had been attempted for at least 50 days; most rats accepting their grafts for that time also kept them when examined more than 100 days after grafting, thus fulfilling stringent criteria for tolerance (17). Rats carrying a healthy first skin graft accepted a second graft put on 15 or more days after the first one; on those occasions when the first graft was later rejected, the second graft was rejected at the same time. The acceptance of second skin grafts served as additional evidence that tolerance had been achieved.

Untreated control rats rejected the allogeneic B/N or W/Fu grafts, the median survival time for 10 B/N rats getting W/Fu grafts being 11.9 ± 0.3 days, and for 24 W/Fu rats getting B/N grafts 10.3 ± 0.4 days.

Skin Grafting.—The skin grafting technique used was slightly modified from one described by Billingham and Silvers (16). Rats were anesthetized with sodium pentobarbital, giving 35 mg/kg body weight. A 2.5 X 2.5 cm piece of allogeneic skin was grafted in a bed prepared by excising the skin down to the deep fascia on the lateral chest wall of the recipient. The rats were then given a plaster bandage, which was removed on day 7 or 8, when the graft cond-
tion was first recorded. New checks of the graft were made daily between the 9th and 14th days, after which the grafts were checked every 2nd day. The bandage was temporarily removed for checking up to the 11th-14th days, when it was discarded.

Target Cells.—Fibroblasts were cultivated from lungs explanted from newborn B/N, W/Fu, and (B/N × W/Fu)F1 rats. The cells were maintained in culture, using Waymouth's medium with 20% fetal calf serum.

Sera.—All test animals were bled at different time points from the tail vein. Sera were separated and stored at −20°C until tested. Control sera were obtained from normal B/N and W/Fu rats and were stored in the same way.

Separation of Blood Lymphocytes.—1.5 ml vol of heparinized blood were drawn from the femoral veins of experimental and control rats. Lymphocytes were separated by centrifugation on cushions of silica gel with different densities, using a technique described by Pertoft et al. (23).

In Vitro Assays of Lymphocyte-Mediated Cytotoxicity.—Blood lymphocytes from B/N and W/Fu rats, inoculated with W/Fu or B/N bone marrow cells as newborns, were tested for ability to destroy W/Fu and B/N fibroblasts, both sets of fibroblasts being simultaneously tested with both types of rats. In some experiments (B/N × W/Fu)F1 fibroblasts were used as well. The previously described microcytotoxicity technique (22) was employed. Lymphocyte doses between 0.75 × 10⁶ and 3 × 10⁶ (occasionally also 0.4 × 10⁶) were tested. There was a minimum of eight replicates per lymphocyte dose. Percentage of target cell numbers after exposure to experimental group lymphocytes was calculated by comparison with groups receiving the same dose of control lymphocytes. The outline of this type of experiment is shown in Table I.

In Vitro Assays of Serum Blocking Activity.—The microcytotoxicity test was also used to search for serum blocking activity, which is defined as the ability of a serum to specifically abrogate target cell destruction by immune lymphocytes. The assays were performed by

| TABLE I |
| Presentation of One Experiment Performed to Test the Cytotoxic Effect of Peripheral Blood Lymphocytes, Using a Microcytotoxicity Assay |

| Lymphocyte donor | no. of remaining attached W/Fu fibroblasts/well (mean ± SE) with indicated no. of lymphocytes | no. of remaining attached B/N fibroblasts/well (mean ± SE) with indicated no. of lymphocytes |
|------------------|-------------------------------------------------|-------------------------------------------------|
|                  | 3 × 10⁶ | 1.5 × 10⁶ | 0.75 × 10⁶ | 0.4 × 10⁶ | 3 × 10⁶ | 1.5 × 10⁶ | 0.75 × 10⁶ | 0.4 × 10⁶ |
| Normal B/N rat   |         |           |           |           |         |           |           |           |
| B/N, no. 20 inoculated with W/Fu cells* | 67.2 ± 1.9 | 63.7 ± 1.5 | 54.1 ± 1.6 | 59.0 ± 1.6 | 36.1 ± 1.3 | 33.7 ± 1.3 |
| B/N, no. 22 inoculated with W/Fu cells* | 45.6 ± 1.3 | 52.4 ± 2.4 | 50.1 ± 2.1 | NT§ | 38.4 ± 0.9 |
| Sensitized B/N rat† | 44.7 ± 1.4 | 51.5 ± 1.4 | 49.8 ± 0.9 | NT | 37.2 ± 1.2 | 36.4 ± 1.2 |
|                  | 31.5 ± 0.9 | 35.0 ± 1.5 | 34.4 ± 1.1 | 37.2 ± 1.2 |

* B/N rats nos. 20 and 22 were neonatally inoculated with W/Fu bone marrow cells and tested at age 42 days. They accepted skin grafts from W/Fu for >98 days and 34 days, respectively.
† Normal adult B/N rat sensitized with a W/Fu skin graft. Blood lymphocytes tested soon after graft rejection.
§ NT, not tested.
incubating target cells with serum for 45 min after which the serum was decanted and lymphocytes added (22). Sera were diluted 1:6 in Eagle’s F12 medium before testing. Each serum was then tested for its ability to block destruction of B/N and W/Fu fibroblasts by lymph node cells from specifically sensitized W/Fu and B/N rats. About one-third of the sera was also tested in combination with (B/N X W/Fu)F1 target cells. Normal B/N and W/Fu sera were always included as controls. One experiment of this type is shown in detail in Table II.

Blocking serum activity was calculated as the ability of a test serum, in comparison with a normal syngeneic serum, to abrogate cell-mediated destruction of the respective target cells, 100% blocking activity meaning that a serum could completely abrogate detectable cytotoxicity.

The statistical significance of blocking serum activity and of destruction of target cells by experimental group as compared with control lymphocytes was calculated by performing Student’s t tests.

Immunized Rats.—As a source of immune lymphocytes when testing sera for blocking activity, lymph node cells were harvested from three types of immunized rats: (a) W/Fu and B/N rats were twice immunized with B/N and W/Fu cells given as 10^7 pooled spleen, thymus, bone marrow, and lymph node cells per rat; (b) W/Fu and B/N adult rats received B/N and W/Fu skin allografts and were used as immune donors after rejection of these grafts; (c) rats from group b were inoculated with allogeneic cells as outlined under a, starting 8-10 days after skin graft rejection.

RESULTS

Cell-Mediated Immunity.—A study was conducted to determine whether blood lymphocytes from rats that had been neonatally inoculated with foreign bone marrow cells could destroy cultivated lung fibroblasts of the respective types, as compared with lymphocytes from normal B/N and W/Fu rats. The experimental outline is shown in Table I. Data on W/Fu rats given B/N cells are presented in Tables III and IV and data on B/N rats given W/Fu cells are shown in Table V.

The majority of rats were tested in vitro before they were skin grafted in order to check for tolerance to the respective allogeneic tissue in vivo. At that time, lymphocytes from 23 of 24 rats were found to be cytotoxic to target cells taken from the strain whose cells were used for the inoculation, and no difference in the degree of reactivity was seen between those rats that later proved to be tolerant and the nontolerant animals. The lymphocyte cytotoxicity was less than that of controls sensitized with skin grafts as adults (but never inoculated neonatally). The latter still had cytotoxic lymphocytes at a dose of 0.75 X 10^5 and 0.4 X 10^5 cells per well, while the former’s lymphocyte effect was only detected when 1.5 X 10^5 (19 of 21 rats) and 3 X 10^5 (23 of 24 rats) lymphocytes were added per well. The lymphocyte suspensions were not cytotoxic when concomitantly tested on syngeneic target cells.

Four rats (nos. 1, 4, 20, 23) were studied when they had carried one tolerated skin graft for 48-55 days and a second one for 4-30 days. All these rats had detectable cell-mediated immunity. This was higher in the rats carrying skin grafts than it had been when the same animals were tested earlier in their life, before skin grafting. It was of the same order of magnitude as in concomitantly
### TABLE II

**Presentation of One Complete Experiment Performed to Test Blocking Serum Activity, i.e., the Ability of Serum to Abrogate the Cytotoxic Effect of Sensitized Lymph Node Cells**

| Target cells | Lymph node cell donor | Serum donor* | no. of remaining attached target cytotoxicity with cells/well (mean ± SE) | Percent blocking |
|--------------|-----------------------|--------------|-------------------------------------------------------------------------|-----------------|
| B/N lung fibroblasts | Normal W/Fu | Normal W/Fu | 43.5 ± 1.3 | 46.0 |
| | Normal B/N | Normal B/N | 40.0 ± 1.1 | 45.0 |
| | B/N 't' W/Fu | Normal B/N | 40.7 ± 1.9 | 49.1 |
| | W/Fu 't' B/N | Normal B/N | 43.5 ± 0.8 | 26.0 |
| | Normal W/Fu | Normal W/Fu | 36.7 ± 1.1 | 43.5 |
| | Normal B/N | Normal B/N | 38.0 ± 1.2 | 4.9 |
| | B/N 't' W/Fu | Normal B/N | 42.8 ± 1.6 | 3.9 |
| | W/Fu 't' B/N | Normal B/N | 38.7 ± 1.7 | 3.0 |
| | W/Fu sensitized to B/N | Normal W/Fu | 23.5 ± 1.0 | 46.0 |
| | Normal B/N | Normal B/N | 22.0 ± 1.0 | 45.0 |
| | B/N 't' W/Fu | Normal B/N | 20.7 ± 0.9 | 49.1 |
| | W/Fu 't' B/N | Normal B/N | 32.2 ± 0.9 | 26.0 |
| | B/N sensitized to W/Fu | Normal W/Fu | 36.6 ± 0.9 | 0.3 (NS) |
| | Normal B/N | Normal B/N | 41.6 ± 1.7 | 2.9 (NS) |
| | B/N 't' W/Fu | Normal B/N | 43.8 ± 1.5 | -0.2 (NS) |
| | W/Fu 't' B/N | Normal B/N | 36.7 ± 1.1 | 3.5 |
| W/Fu lung fibroblasts | Normal W/Fu | Normal W/Fu | 30.1 ± 1.0 | 46.0 |
| | Normal B/N | Normal B/N | NT | 46.0 |
| | B/N 't' W/Fu | Normal B/N | 46.4 ± 1.7 | 6.9 (NS) |
| | W/Fu 't' B/N | Normal B/N | 29.4 ± 1.1 | -4.4 (NS) |
| | Normal B/N | Normal W/Fu | 33.1 ± 1.1 | 3.4 (NS) |
| | Normal B/N | Normal B/N | 46.5 ± 1.8 | 6.9 (NS) |
| | B/N 't' W/Fu | Normal B/N | 43.7 ± 1.6 | -4.4 (NS) |
| | W/Fu 't' B/N | Normal B/N | 41.1 ± 1.3 | -4.4 (NS) |
| | W/Fu sensitized to B/N | Normal W/Fu | 29.1 ± 1.0 | 3.4 (NS) |
| | Normal B/N | Normal B/N | NT | NT |
| | B/N 't' W/Fu | Normal B/N | 43.2 ± 1.5 | 6.9 (NS) |
| | W/Fu 't' B/N | Normal B/N | 30.7 ± 0.9 | -4.4 (NS) |
| | B/N sensitized to W/Fu | Normal W/Fu | 21.4 ± 0.7 | 35.4 |
| | Normal B/N | Normal B/N | 29.5 ± 1.1 | 36.6 |
| | B/N 't' W/Fu | Normal B/N | 34.7 ± 1.2 | 20.6 |
| | W/Fu 't' B/N | Normal B/N | 25.2 ± 1.0 | 38.7 | 43.7 |

All sera were diluted 1:6.

* Sera tested from normal rats (for controls), from B/N rats inoculated with W/Fu cells as newborns for tolerance induction (B/N 't' W/Fu) and from W/Fu rats similarly inoculated with B/N cells (W/Fu 't' B/N). The tolerant serum donors studied in this experiment were no. 23 (B/N 't' W/Fu) and no. 3 (W/Fu 't' B/N) that are further described in Tables IV and V. ‡ NT = not tested. § Probabilities that differences between experimental and control groups are due to chance are indicated: * P < 0.01, † P < 0.001, NS = not significant (P > 0.05).
### TABLE III

**Cell-Mediated Immunity in W/Fu Rats That Received B/N Bone Marrow Cells as Newborns**

| W/Fu test rat | Status of B/N skin graft | Cell-mediated immunity* |
|---------------|--------------------------|-------------------------|
|               | First graft | Second graft | Time after birth when tested | Percent cytotoxicity by indicated no. of test lymphocytes on B/N fibroblasts |
|               | Age of test rat when grafted | Survival of skin graft | Age of test rat when grafted | Survival of skin graft | days | days | days | 3 x 10^5 | 1.5 x 10^6 | 0.25 x 10^6 | 4 x 10^5 |
|               | Sex | Age | no. | Sex | Age | no. | Sex | Age | no. | 3 x 10^5 | 1.5 x 10^6 | 0.25 x 10^6 | 4 x 10^5 |
| 9  M 42 | 42 | 74 | 15 | 35 | 28.1| 24.7| NT | NT |
| 11  F 42 | 10 | 74 | 7 | 35 | 37.9| 33.8| NT | NT |
| 12  F 42 | 10 | 74 | 7 | 35 | 38.9| 33.8| NT | NT |
| 13  M 42 | 10 | 74 | 7 | 35 | 34.6| 41.1| NT | NT |
| 14  M 37 | >147 | 69 | >115 | 44 | 20.9| 23.1| 3.2(NS) | NT |
| 15  M 37 | >147 | 69 | >115 | 44 | 26.9| 30.1| 3.2(NS) | NT |
| 16  M 37 | 12 | 69 | 7 | 44 | 31.8| 32.1| 6.6(NS) | NT |
| 18  F 37 | 12 | 69 | 7 | 44 | 31.8| NT| 3.7(NS) | NT |
| 30  F 40 | 122 | 69 | 90 | 44 | 32.2| NT| 3.7(NS) | NT |
| 31  M 52 | >128 | 82 | >88 | 40 | 42.9| 33.1| 9.6(NS) | NT |
| 33  M 52 | >128 | 82 | >88 | 40 | 40.5| 30.1| 6.8(NS) | NT |
| 34  M 52 | >128 | 82 | >88 | 40 | 40.5| 30.1| 13.8(NS) | NT |
| 35  M 52 | >128 | 82 | >88 | 40 | 47.8| NT| 6.2(NS) | NT |
| 38  M 46 | >113 | 86 | >73 | 147 | 3.0(NS)| NT| NT | NT |
| 40  M 46 | >113 | 86 | >73 | 147 | 4.6(NS)| -20.1(NS)| NT| NT |
| 41  M 46 | >113 | 86 | >73 | 147 | 10.8| 21.1| 1.6(NS) | NT |
| 42  M 46 | >113 | 86 | >73 | 143 | 68.8| 15.4(NS)| NT| NT |
| 50  M 45 | >113 | 90 | >98 | 140 | 30.6| -3.1(NS)| NT| NT |
| 51  M 56 | >100 | 113 | >43 | 140 | 21.1| 30.0| NT| NT |
| 52  M 56 | >100 | 109 | >47 | 47 | 30.1| 24.4| 16.7§| NT |
| 53  F 55 | Died | - | - | 47 | 33.3| 23.7| -20.8(NS)| NT |
| 54  F 55 | Died | - | - | 47 | 8.7(NS)| 12.5(NS)| 4.9(NS)| NT |
| 55  F 55 | Died | - | - | 47 | 31.9| 26.9| 3.9(NS)| NT |
| 57  M 52 | Died | - | - | 47 | 29.7| 15.1(NS)| 7.71(NS)| NT |
| 60  M 53 | >100 | 105 | >47 | 147 | 18.6| 40.0| NT| NT |

*The cytotoxic effect was tested on B/N and, in some experiments, also on (B/N X W/Fu) F1 target cells. It was calculated from comparisons with groups receiving the same doses of lymphocytes from normal (noninoculated) W/Fu rats. Probabilities that differences between experimental and control groups are due to chance are indicated: § P < 0.05, ¶ P < 0.01, II P < 0.001, NS = not significant (P > 0.05). The same lymphocyte suspensions were also tested on W/Fu fibroblasts, on which they had no significant cytotoxic effects.

**Skin graft sensitizer**

| W/Fu test rat | Status of B/N skin graft | Cell-mediated immunity* |
|---------------|--------------------------|-------------------------|
|               | First graft | Second graft | Time after birth when tested | Percent cytotoxicity by indicated no. of test lymphocytes on B/N fibroblasts |
|               | Age of test rat when grafted | Survival of skin graft | Age of test rat when grafted | Survival of skin graft | days | days | days | 3 x 10^5 | 1.5 x 10^6 | 0.25 x 10^6 | 4 x 10^5 |
| 7  days | after graft rejection | 32.1| 46.2| 32.7| 28.7| NT |

**Skin graft rejection**

1. NT = not tested.
TABLE IV

**Correlation of Cell-Mediated Immunity and Blocking Serum Activity with Skin Graft Survival in W/Fu Rats That Received B/N Bone Marrow Cells as Newborns**

| W/Fu test rat | Status of B/N skin graft | Cell-mediated immunity† | Blocking serum activity** in percent at different time points after birth |
|---------------|--------------------------|-------------------------|---------------------------------------------------------------------|
|               | First graft | Second graft | Percent cytotoxicity by indicated no. of test lymphocytes on B/N fibroblasts | Time after birth when tested | 3 × 10^5 | 1.5 × 10^6 | 0.75 X 10^8 |
| no. | Sex | Age of test rat when grafted | Survival of skin graft | Age of test rat when grafted | Survival of skin graft | 39 | 65 | 81 | 87 | 91 | 119 | 133 | 147 | 163 | 183 |
| 1 | M | days | days | >160 | 72 | >142 | 102* | 27.3% | 7.2 (NS) | 39.4% | 29.0% | 57.9% | 49.2% | NT | 100.0% | 51.2% | NT | 100.0% | 100.0% | NT | 71.8% |
| 2 | M | days | days | >160 | 86 | >128 | 191 | 23.5% | 9.8 (NS) | 42.0% | 47.9% | 24.4% | 100.0% | 62.4% | 58.9% | NT | NT | NT | NT | NT | NT |
| 3 | M | 54 | 66 | 99 | 18 | NT | NT | NT | NT | 100.0% | 67.6% | NT | NT | NT | NT | NT | NT | NT | NT |
| 4 | M | 54 | 96 | 86 | 63 | 102* | 23.4% | 36.3% | 20.0% | 40.2% | 58.8% | 100.0% | 94.4% | 58.0% | NT | 100.0% | -20.7 | -34.5 | NT |
| 6 | M | 54 | 26 | 86 | 9 | 102* | 43.5% | 37.8% | 26.8% | 38.1% | 2.8 (NS) | -22.4 | -22.4 | -69.2 | NT | 20.0 (NS) | 16.3 (NS) | 24.8 (NS) | NT |
| 7 | F | 54 | >160 | 86 | >128 | 191 | 11.1 (NS) | NT | NT | 100.0% | 90.3% | 91.6% | NT | NT | NT | 50.2% | 100.0% | 90.1% | NT |

*The tests for cell-mediated immunity were done 48 days after first grafting. NT = not tested.
†The cytotoxic effect was tested on B/N and, in some experiments, also on (B/N × W/Fu)F1 target cells. It was calculated from comparisons with groups receiving the same doses of lymphocytes from normal (nonimmunized) W/Fu rats. Probabilities that differences between experimental and control groups are due to chance are indicated: §P < 0.05, §§P < 0.01, §§§P < 0.001, NS = not significant (P > 0.05). The same lymphocyte suspensions were also tested on W/Fu fibroblasts, on which they had no significant cytotoxic effects.

**Blocking activity was defined as illustrated in Table II, by the ability of a 1:5 diluted serum (as compared with control serum from syngenic rats) to depress destruction of B/N fibroblasts by specifically immune W/Fu lymph node cells.
TABLE V
Correlation of Cell-Mediated Immunity and Blocking Serum Activity with Skin Graft Survival in B/N Rats That Received W/Fu Bone Marrow Cells as Newborns

| B/N test rat no. | Sex | Status of W/Fu skin grafts | Cell-mediated immunity† | Blocking serum activity** in percent at different time points after birth |
|------------------|-----|---------------------------|-------------------------|--------------------------------------------------|
|                  |     | First graft               | Time after birth when tested | Percent cytotoxicity by indicated no. of test lymphocytes on W/Fu fibroblasts | days |
|                  |     | Age of test rat when grafted | Age of survival of skin graft | 3 X 10⁶ | 1.5 X 10⁶ | 0.75 X 10⁶ | 0.4 X 10⁶ | 38 | 46 | 65 | 70 | 74 | 87 |
| 20               | F   | 50 days                    | 100 days                  | 42 | 105 | 32.1 | 17.7 | 7.4 (NS) | NT | 51.9 | NT | 60.2 | 47.2 | 66.6 | 87.0 |
| 22               | F   | 50 days                    | 100 days                  | 42 | 105 | 33.5 | 19.2 | 7.9 (NS) | NT | 45.7 | 60.1 | 58.8 | 56.3 | 4.7 (NS) | 8.1 |
| 23               | M   | 50 days                    | 101 days                  | 42 | 105 | 34.0 | 16.4 | 3.7 (NS) | NT | 20.4 | 61.4 | 43.7 | 26.9 | NT | 37.3 |
| 24               | M   | 50 days                    | 101 days                  | 42 | 105 | 34.1 | 29.0 | 21.4 | NT | 27.5 | 59.1 | 37.7 | 23.6 (NS) | NT | NT |
| 25               | M   | 50 days                    | 102 days                  | 42 | 105 | 34.3 | 20.4 | 5.0 (NS) | NT | 33.9 | 34.7 | 24.6 (NS) | 16.3 (NS) | NT | NT |
| 26               | M   | 50 days                    | 101 days                  | 42 | 105 | NT* | NT | NT | NT | 26.8 | NT | 9.0 (NS) | -28.9 | 20.0 (NS) | NT |

* NT = not tested.
† The cytotoxic effect was tested on W/Fu and, in some experiments, also on (B/N X W/Fu)F₁ target cells. It was calculated from comparisons with groups receiving the same doses of lymphocytes from normal (noninoculated) B/N rats. Probabilities that differences between experimental and control groups are due to chance are indicated: †P < 0.05, †P < 0.01 |
P < 0.001, NS = not significant (P > 0.05). The same lymphocyte suspensions were also tested on B/N fibroblasts, on which they had no significant cytotoxic effects.
** Blocking activity was defined as illustrated in Table II by the ability of a 1:5 diluted serum (as compared with control serum from syngeneic rats) to depress destruction of W/Fu fibroblasts by specifically immune B/N lymph node cells.
tested rats nos. 6 and 22, which had rejected their skin grafts in spite of the fact that they had received allogeneic cells as newborns.

13 rats were also tested after they had carried tolerated skin grafts for 84-143 days. 9 of these rats had a significant cell-mediated cytotoxicity with $3 \times 10^5$ lymphocytes per well, while only 4 of the 13 rats were reactive with $1.5 \times 10^4$ lymphocytes per well. This indicates that the cell-mediated immunity was lower when tested late after tolerance induction than it was when the tests were performed closer in time to the neonatal inoculation (and skin grafting). Tests on rats nos. 1, 14, and 33 illustrate this point.

The number of circulating lymphocytes in the neonatally inoculated animals varied between 4.2 and $8.0 \pm 10^6$/ml, when determined before the first skin grafting. It was not different from that of control rats.

Blocking Serum Activity.—An experiment performed to search for blocking serum activity in neonatally inoculated rats is presented in detail in Table II, and our whole material is summarized in Tables IV and V.

Sera from rats inoculated neonatally with allogeneic cells and capable of accepting skin grafts of the respective types for prolonged periods of time could block the cytotoxic effect of sensitized lymphocytes. The blocking effect was specific: Sera from W/Fu rats tolerant to B/N cells blocked destruction of B/N but not of W/Fu target cells (and vice versa). No significant difference was seen, under the conditions of our experiments, dependent on whether control serum from W/Fu or B/N rats was used with a given set of target cells. A specific blocking effect was also detected in tests on (B/N X W/Fu)F1 hybrid fibroblasts.

A remarkable correlation was found between serum blocking activity in vitro and skin graft survival in vivo (Tables IV and V). Sera from all rats that carried allogeneic skin grafts over prolonged periods of time were blocking before grafting and remained so as long as the grafts were kept (the latest serum sample tested was taken 129 days after grafting). Three rats (nos. 6, 25, and 26) had sera that were blocking both before and shortly after grafting, but lost the blocking activity within 7-10 days before rejection; these rats rejected their grafts after 20-27 days. Sera from rat no. 4, which carried its first skin graft for 96 days, were blocking 17 days (but not 3 days) before graft rejection. Sera from rats that had rejected their grafts were never blocking in the dilution tested (1:6).

DISCUSSION

At least two conclusions can be drawn from the present observations. First, they show that rats behave similarly to the previously studied mice (6), in that most neonatally inoculated animals that accept foreign skin grafts of the respective strains over prolonged periods of time have lymphocytes cytotoxic to target cells carrying the tolerated antigens and in that they have a blocking serum activity capable of canceling this cytotoxicity. It may not necessarily have been so, since lymphocytes from tolerant rats have been shown to be
specifically nonreactive in mixed leukocyte tests (18, 19). Second, our findings point towards a correlation between the in vitro parameters measured (lymphocyte-mediated cytotoxicity and blocking serum activity) and the in vivo situation, in that blocking serum activity was seen to disappear before the rejection of previously accepted skin grafts in those rats in which such rejections occurred. The blocking factors are believed to be antigen-antibody complexes or antibodies, rather than antigens, since allogeneic serum from the tolerated strain did not block under the conditions of our tests; no studies on the nature of the blocking factors in tolerant rats have been conducted, however. Neither have we studied the nature of the “killer” cells detected in the tolerant animals (B?, T?, macrophages?) or their mechanisms of action. One cannot exclude, therefore, that animals with cytotoxic blood lymphocytes are deficient with respect to (some of) those T cell clones that are capable of reacting against the tolerated antigens.

The cytotoxic effect of lymphocytes from tolerant animals decreased in strength as the time interval between neonatal inoculation (and skin grafting) and the test increased, and 4 of 13 rats that were carrying skin grafts for 84 or more days were nonreactive (in the highest dose tested) while lymphocytes from 23 of 24 rats tested within 2 mo after birth had a cytotoxic effect. A similar decrease of detectable cell-mediated immunity has been seen in some human patients carrying allogeneic kidney grafts (24), to which they initially reacted, while on the other hand, it was not observed in radiation-induced canine chimeras (10) or in tetraparental mice (8). One possibility is that the loss (decrease) of cell-mediated immunity represents a more complete form of tolerance which is qualitatively different from that involving coexistent cellular immunity and blocking serum activity. This tolerance may, indeed, be due to the elimination of “forbidden clones.” It may also, however, be due to a more effective blocking of otherwise reactive cell clones making cytotoxicity undetectable under the experimental conditions used so far. Whichever alternative is correct (or even if none of them is), one must realize that, in the present studies, those rats that lacked lymphocyte reactivity when tested late after tolerance induction, indeed had such reactivity earlier and that this reactivity (presumably blocked by serum factors in vivo) was fully compatible with survival of (“tolerance to”) skin grafts. It remains to be studied whether the blocking serum activity disappears with time in those rats that have lost their cell-mediated reactivity.

Although our data fit the hypothesis that a blocking serum activity, as measured in vitro, plays a role in the maintenance of allograft tolerance in vivo, it is too early to arrive at conclusions as to the importance of that role as compared with other mechanisms until more is known about how the in vitro observations correlate with the nonreactive state in vivo. For example, one needs to know whether there are changes in blocking serum activity and in the level of lymphocyte-mediated cytotoxicity upon transfer of nonimmune, non-
tolerant lymphocytes (or specifically immune lymphocytes) in order to break the tolerant state. One also wants to know whether tolerance can be transferred if large enough quantities of serum are given so that samples taken from animals receiving tolerant serum will block lymphocyte cytotoxicity when tested in vitro; when experiments of this type were performed with syngeneic tumors carrying specific antigens, using sera from tumor-bearing animals, it was indeed possible to show that sera blocking lymphocyte reactivity in vitro could enhance tumor growth in vivo (25). Furthermore, one wonders what information concomitantly performed mixed leukocyte assays will give on the same rats. It is not unlikely that an animal may be found to be nonreactive with that assay but still reactive in the microcytotoxicity test, because of the different cellular functions (and, possibly, different cellular clones) studied by the two tests. Answers to these questions may be obtained by studying tolerant rats with presently available in vitro assays, particularly since the same animals can be tested repeatedly. Finally, we want to emphasize, once more, that we have used the term “tolerance” in a strictly operational sense: rats were referred to as tolerant if they retained their skin grafts for at least 50 (commonly more than 100) days. The possibility remains that rats in which tolerance is induced according to some protocol different from the one we followed, receiving, e.g. much larger inocula of foreign cells neonatally, will behave differently (e.g. like our rat no. 38 in Table III that lacked detectable lymphocyte cytotoxicity). Even if it would be so, however, this would not detract from the interest of the demonstration that rats can retain skin grafts over long periods of time (permanently?) in the presence of sensitized lymphocytes and that blocking serum factors appear to play an important role in making this possible.

SUMMARY

W/Fu rats were neonatally inoculated with bone marrow cells from B/N rats and vice versa. Of the inoculated rats, some were capable of accepting a foreign (B/N or W/Fu) skin graft over the period of observation (i.e. for more than 100 days), while other rats rejected their skin grafts as early as control animals (within 8-12 days) or after a prolonged period of acceptance (20-96 days).

Using a microcytotoxicity test, it could be shown that both those rats that rapidly rejected skin grafts and those that kept their grafts during the observation period had lymphocytes capable of destroying cultivated allogeneic cells from the respective strains with whose cells the rats had been inoculated as newborns. The degree of lymphocyte reactivity decreased upon time, so that 4 of 13 rats that had carried “tolerated” skin grafts over more than 84 days had lymphocytes which were nonreactive in the highest dose tested, and the degree of reactivity in the other 9 rats was less than seen early after tolerance induction.

Rats that were capable of accepting skin grafts over prolonged periods of
time had sera that could specifically block lymphocyte-mediated cytotoxicity, while sera from rats that had rejected their grafts did not block. Sera from rats that rejected their skin grafts after 20–96 days lost the blocking activity 3–10 days before rejection.

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