

Brief Definitive Reports

THYMUS-DEPENDENT LYMPHOCYTES IN TISSUE SECTIONS OF REJECTING RAT RENAL ALLOGRAFTS*

By C. M. BALCH†, C. B. WILSON, S. LEE, AND J. D. FELDMAN

(From the Department of Experimental Pathology, Scripps Clinic and Research
Foundation, La Jolla, California 92037)

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Thymus-dependent (T) lymphocytes have recently been associated with a number of functions among which are in vitro cytotoxic activities to cells bearing histoincompatible antigens. In vivo, the presence of T lymphocytes has been detected by immunofluorescence in lesions of delayed hypersensitivity (1) and Marek's disease (2). T lymphocytes have also been shown to be active or present in tissues undergoing rejection by applying less direct means of identification, e.g., by measuring cytotoxicity against tumor target cells (3, 4) or by tracing the localization of labeled cells (5). In this paper we report the flow of T lymphocytes into cellular infiltrates of rejecting renal allografts. T lymphocytes were stained in tissue sections by fluorescein-labeled horse antisera specific for rat T lymphocytes.

Materials and Methods

Rats.—18 Lewis (L) rat renal allografts and 12 Brown Norway (BN) rat renal isografts were transplanted heterotopically into BN recipients, as previously described (6). Bladder anastomosis was not performed and both host kidneys remained in place. Recipient and donor rats were males, 4–8 mo of age, and weighed 250–350 g. At daily intervals for 6 days after transplantation, groups of three renal allograft and two renal isograft recipients were sacrificed. One-half of an excised renal transplant was snap frozen in liquid nitrogen and stored at -30°C , while the other half was fixed in Bouin's solution for routine histological study. The non-transplanted donor kidney and a host kidney were excised at the time the grafts were removed, were prepared the same as the grafts, and served as additional controls.

Reagents.

Fluoresceinated IgG of horse antirat thoracic duct cell antiserum (HART-F) and normal horse serum (NHG-F): The properties of HART-F and its specificity for T cells have been described elsewhere (7). Briefly, horse antirat thoracic duct cell antiserum¹ was inactivated at 56°C for 45 min and an immunoglobulin (Ig) fraction prepared by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by passage through a DEAE-Sephadex A-50 column. An Ig fraction of normal horse serum was similarly prepared. 10 mg of each Ig fraction was conjugated with 2.5 mg of fluorescein

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† Supported by National Institutes of Health Training Grant GM01924-04. Present address: Department of Surgery, University of Alabama, Birmingham, Ala. 35233.

¹ This was generously supplied by Dr. George White, The Upjohn Co., Kalamazoo, Mich. It was an antiserum taken from a horse that had been repeatedly injected with rat thoracic duct cells.

isothiocyanate (FITC; Baltimore Biological Laboratories, Division of BioQuest, Cockeysville, Md.) and eluted from a Sephadex G-25 column. The labeled fractions had F/P ratios of 4 to 6. The fluoresceinated reagents were each absorbed sequentially with $\frac{1}{5}$ to $\frac{1}{10}$ packed volume of rat erythrocytes, kidney cells, fetal liver, bone marrow, glutaraldehyde-insolubilized rat serum, and glutaraldehyde-insolubilized rat kidney homogenate (8). An aliquot of HART-F was further absorbed two times with rat thymocytes and used as a control reagent for specificity tests. HART-F stained >95% viable thymocytes, <2% bone marrow cells, and eliminated cytotoxic activities against in vitro target cells (7).

Tissue Immunofluorescence.—Tissue sections (5–10 mm on edge and 2 μ m in thickness) from frozen transplanted and control kidneys were cut in the cryostat at several levels of the renal cortex and placed on glass slides. The sections were then soaked in phosphate-buffered saline (PBS), 5 min, fixed in ether-ethanol, 1:1 for 10 min, followed by 95% ethanol, 20 min, and washed three times with PBS. 10–20 μ l of fluoresceinated reagent was placed on the tissue section for 40 min in a humidified box at 25°C. The slides were washed three times with PBS and mounted with cover slips in 0.01 M Tris-buffered glycerin, pH 9.5. The slight decrease in intensity of staining noted in fixed sections was compensated for by the increased clarity over that of their unfixed counterparts.

The tissue sections were examined in a Zeiss RA microscope equipped with dark-field condenser, FITC interference exciting filter,² 500 nm barrier filter, and illuminated from an HBO 200 W mercury light source.

Anti-T Lymphocyte Antiserum Treatment of Renal Allograft Recipients.—A group of renal allograft recipients received 1 ml of rabbit antirat thymocyte antiserum (RART) or normal rabbit serum (NRS) intravenously every other day commencing 2 days before grafting. RART-treated recipients were sacrificed 4 days after grafting (two animals) or at 7 days (four animals). Two recipients treated with NRS were sacrificed at 7 days posttransplantation. Kidneys at autopsy were handled as described above.

RART was prepared and absorbed to render it specific for T cells as described elsewhere (7, 9). Briefly, rabbits received 10^9 rat thymocytes intravenously two times at a 2 wk interval and were exsanguinated 1 wk after the second immunization. The antiserum was inactivated at 56°C for 45 min and absorbed as described above for HART-F. The unfractionated absorbed RART antiserum killed aggressor cells in cell-mediated cytotoxicity tests, spleen cells responding to phytohemagglutinin and concanavalin A but did not kill antibody-producing cells in a Jerne plaque assay. When labeled with fluorescein it stained cells in the same fashion as HART-F. NRS obtained from these same rabbits before immunization was heat inactivated, absorbed two times with rat kidney and with rat RBC, and used as a control reagent.

RESULTS

Specificity of Reagents.—To demonstrate the specificity of HART-F for rat T lymphocytes in tissue sections, viable suspensions of thymus cells, bone marrow, or peritoneal exudate cells were injected under the capsule of normal kidneys. The kidneys were immediately excised, snap frozen in liquid nitrogen, and sectioned tangentially to include the area of subcapsular lymphoid cells. HART-F stained virtually all thymocytes and only a few bone marrow or peritoneal exudate cells in these tissue sections. Positive cells showed a complete ring stain around their surfaces without cytoplasmic or nuclear staining. Since it was not possible to enumerate accurately the proportion of stained to unstained cells in tissue sections, aliquot of the same cell populations injected under the renal capsule were stained as viable cell suspensions with HART-F and examined in vitro. 98% of thymocytes and 2% of bone marrow cells were fluorescent positive. Large cells in peritoneal exudate suspensions, presumably macrophages, did not stain, and one-half to two-thirds of the small cells, presumably lymphocytes, were stained. In both cell suspensions and tissue sections, NHG-F and HART-F absorbed with thymus cells failed to stain any lymphoid cells.

Renal Allografts.—In the light microscope, cellular accumulations were

² Optisk Laboratorium, Lyngly, Denmark.

visible around a few vessels 24 h after grafting and increased progressively up to 6 days. The histologic and cytologic details were similar to the descriptions previously reported (10).

In the fluorescent microscope stained cells were seen in the perivascular spaces of about 25% of the medium and small vessels present in section and were also scattered in the interstitium as early as 24 h after grafting (Fig. 1). By the 2nd–3rd days, stained cells increased to several hundred and more per section in infiltrates, which encompassed almost all vessels and which were focally distributed in the interstitium and circumferentially around Bowman's capsule. A few stained cells were occasionally seen in glomeruli. Maximal perivascular infiltration of stained cells occurred on day 3 or 4, while the interstitial infiltrate became progressively diffuse on days 4, 5, and 6 after grafting (Fig. 2). The infiltrates contained increasing numbers of unstained cells after the 4th

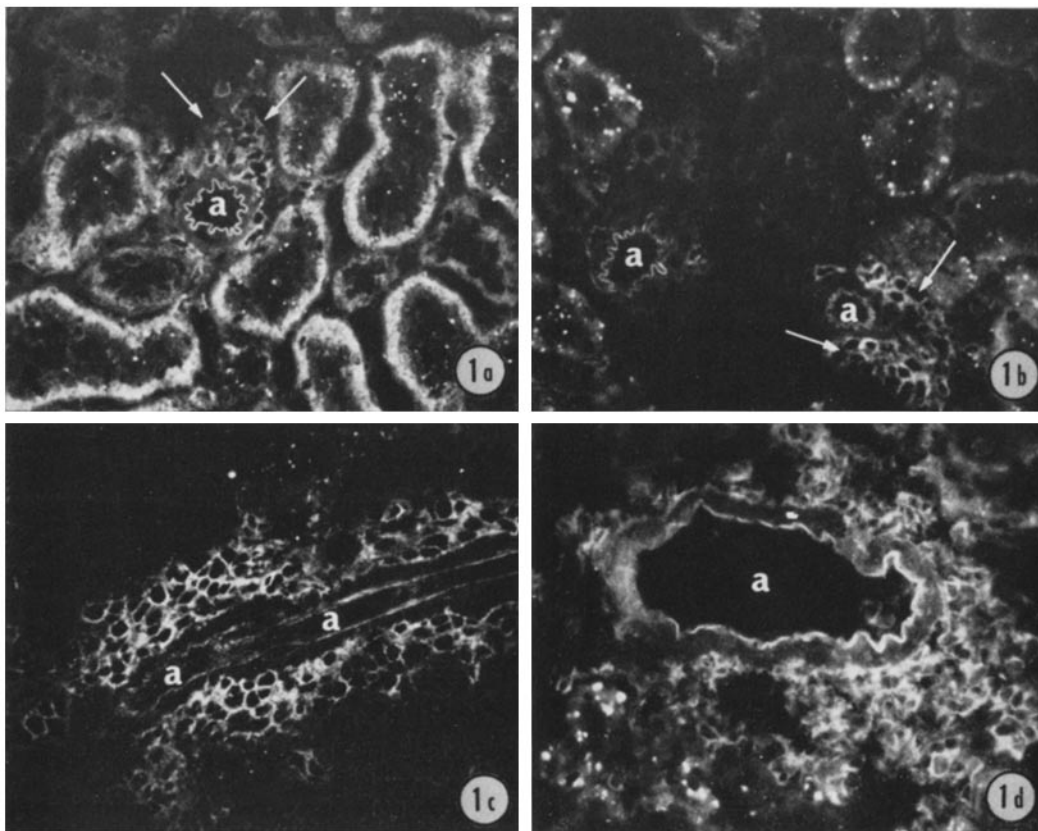


FIG. 1. Cells with positive surface outlines (*arrows*) are seen clustered about some, but not all small arteries (*a*) in L renal allografts 1 day after transplantation to a BN recipient (1 *a*, 1 *b*). 2 days after transplantation the numbers of stained cells visualized surrounding arteries and in the interstitium have increased (1 *c*, 1 *d*). (Fluorescein isothiocyanate-conjugated horse antirat thoracic duct serum [HART-F]). Original magnification $\times 250$.

day. No fluorescent-positive cells were identified in these tissue sections using NHG-F or HART-F absorbed with thymocytes.

Accurate enumeration of total cells and green-stained cells infiltrating a section was not feasible. The findings described above were quite consistent in each group of three allografts, and the magnitude of increasing total cellular and T-cell accumulations was readily discerned from day 1 to day 6.

Renal Isografts, Nontransplanted Control Kidneys, Nonspecific Inflammatory Renal Lesions.—Renal isografts examined up to 6 days after grafting and the nontransplanted control kidneys contained a few isolated T cells in the interstitium as well as occasional small focal accumulations in the interstitium or perivascular areas that appeared to correspond with mononuclear cell infiltrations observed in the light microscope (Fig. 3 *a*).

Nonspecific inflammatory renal lesions were created by injecting xylene into

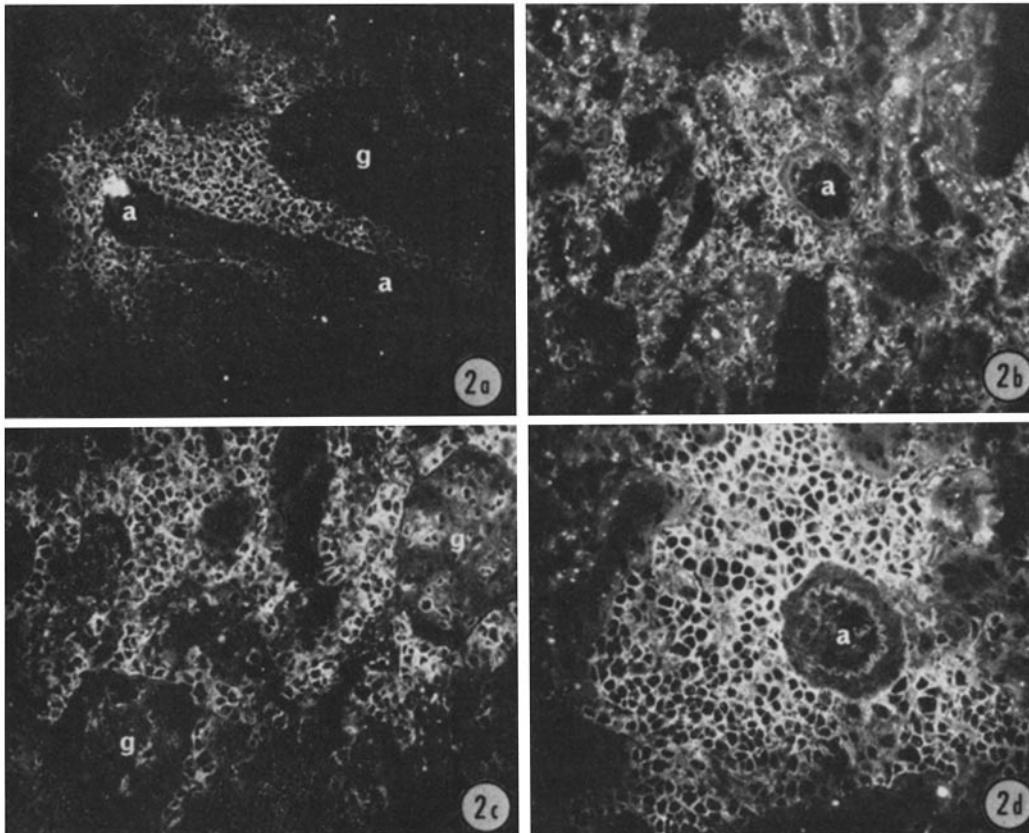


FIG. 2. Extensive infiltrates of positively stained cells are seen surrounding arteries (*a*), in the interstitium, and to a lesser degree glomeruli (*g*) of L rat allografts 4 days after placement in BN recipients. (HART-F) Original magnification: 2 *a*, 2 *b* $\times 160$; 2 *c* $\times 250$; 2 *d* $\times 400$.

a kidney or by multiple punctures with a sterile needle. After 48 h, the kidneys were excised and examined by light and immunofluorescence microscopy. The inflammatory lesions seen by light microscopy contained stained cells by tissue immunofluorescence in a scattered distribution without focal interstitial or perivascular accumulations.

Renal Allografts in Hosts Treated with RART.—4 days after transplantation, renal allografts from RART-treated recipients contained stained cells in the perivascular areas of the cortex that were significantly fewer than the numbers found in untreated renal allografts, i.e., the magnitude of infiltration of stained cells was approximately equal to that in an unmodified allograft 1 or 2 days after grafting. 7 days after transplantation, in hosts given RART, the renal grafts were grossly and histologically viable. In these specimens stained cells were present in numbers approximately equal to those of a 2-day-untreated renal allograft. Stained cells were scattered in the interstitium or present in focal accumulations, but generally did not accumulate as large clusters or masses around blood vessels (Fig. 3 *b*). By comparison, in recipient rats treated with NRS, control allografts were nonviable at 7 days and were diffusely infiltrated with cells.

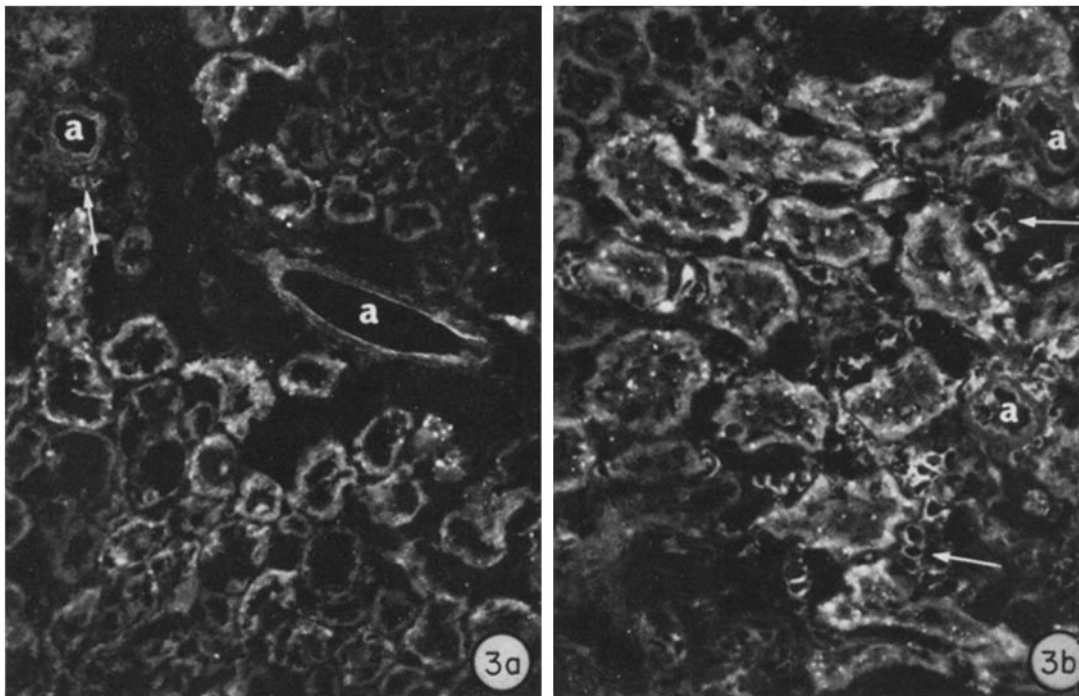


FIG. 3. Only occasional positively stained cells are seen (*arrows*) even after 6 days in a L renal isograft (3 *a*). Rabbit antirat thymocyte antiserum (RART) greatly reduced the numbers of positively staining cells (*arrows*) found in a 7 day L to BN allograft (3 *b*); the infiltration is comparable to a 2 day unmodified allograft. (*a*) indicates arteries. (HART-F) Original magnification: 3 *a* \times 160; 3 *b* \times 250.

DISCUSSION

The reagents and controls used in these studies indicate that the fluorescein-stained cells present in rejecting renal allografts are T lymphocytes. The evidence for this is: (a) HART-F in vitro stains >95% thymocytes, <2% bone marrow elements, and 30–60% of the lymphocytes in spleen and lymph nodes (7); (b) absorption of HART-F with thymocytes eliminates staining of all cells; (c) spleen and lymph node populations incubated in HART and complement (C) at 37°C lost several activities associated with T lymphocytes, e.g., cell cytotoxicity for target cells, response to mitogens, graft rejection (7, 9); (d) HART-F does not stain IgG-bearing lymphocytes nor does it destroy, with C, their capacity to make plaque-forming cells to erythrocytes (7). We conclude, then, that the stained cells are T lymphocytes.

In our renal allografts T cells are detectable 24 h after grafting and reach a maximum within 3–4 days. The proportion of T cells then diminish despite the progression of inflammation and destruction in the kidney up to day 6. T cells are also found in renal isografts and in areas of nonspecific inflammation, although in much smaller numbers than those of rejecting grafts. They have also been seen in the mononuclear interstitial infiltrates present in autologous immune complex glomerulonephritis of rats (Wilson, C. B., unpublished observations) and in this lesion the stained cells are immunologic participants but not cytotoxic elements. From these observations we conclude that the mere presence of T lymphocytes in tissues does not necessarily signify an immunologic reaction. This is to be expected since lymphocytes, and particularly T lymphocytes, are migrant elements and since there are subsets of T lymphocytes each with different functions (11–13).

Our data, however, from grafted rats treated with RART, indicate that some, if not all, T lymphocytes in rejecting kidneys are immunologically reactive agents, either cytotoxic cells attacking histoincompatible tissues or proliferating cells responding to histoincompatible antigens, or both. RART reduced significantly the total number of cells and the number of T lymphocytes infiltrating allografts and also prolonged viability and survival of grafts. Since RART has the same specificities for T cells as does HART, we conclude that elimination of most of the T lymphocytes from the kidney helps to decrease tissue destruction and prolong survival of the graft, and therefore many of the T lymphocytes are cytotoxic cells reacting to foreign antigens. Although staining of T lymphocytes in tissues distinguishes one set of lymphocytes from other sets, the studies do not separate subsets of T lymphocytes from each other, nor do they reveal how other agents, for example, polymorphs, macrophages, antibodies et al., interact in the complex process of rejection.

SUMMARY

Lewis kidneys were grafted into BN recipients and examined at daily intervals up to 6 days after grafting with immunofluorescent reagents. A horse anti-serum specific for T lymphocytes revealed an increasing number of T lymphocytes in the cellular infiltrates of rejecting allografts. These were detectable 1

day after grafting, reached a maximum 3 days later, and were relatively diminished at 6 days. In control isografts and nonimmunological inflammations of kidney, a small number of dispersed T lymphocytes was seen. A rabbit antirat thymocyte antiserum, given to allografted BN rats, prolonged survival of the grafts and decreased the cellular infiltrate and the number of T lymphocytes in the infiltrates. We conclude that in graft rejection there is a flow of T lymphocytes into areas of tissue damage and these T lymphocytes are immunologically reactive to graft antigens.

REFERENCES

1. Williams, R. M., and B. H. Waksman. 1969. Thymus-derived cells in the early phase of delayed tuberculin reactions. *J. Immunol.* **103**:1435.
2. Rouse, B. T., R. J. H. Wells, and N. L. Warner. 1973. Proportion of T and B lymphocytes in lesions of Marek's disease: theoretical implications for pathogenesis. *J. Immunol.* **110**:534.
3. Freedman, L. R., J.-C. Cerottini, and K. T. Brunner. 1972. In vivo studies of the role of cytotoxic T cells in tumor allograft immunity. *J. Immunol.* **109**:1371.
4. Rouse, B. T. and H. Wagner. 1972. The in vivo activity of in vitro immunized mouse thymocytes. II. Rejection of skin allografts and graft-vs-host activity. *J. Immunol.* **109**:1282.
5. Lance, E. M., and S. Cooper. 1972. Homing of specifically sensitized lymphocytes to allografts of skin. *Cell. Immunol.* **5**:66.
6. Lee, S. 1967. An improved technique of renal transplantation in the rat. *Surgery.* **61**:771.
7. Balch, C. M., and J. D. Feldman. Thymus dependent (T) lymphocytes in the rat. *J. Immunol.* In press.
8. Avrameas, S., and T. Ternynck. 1969. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents. *Immunochemistry.* **6**:53.
9. Balch, C. M., and J. D. Feldman. Origin and development of rat thymus-dependent (T) lymphocytes. *J. Immunol.* In press.
10. Feldman, J. D., and S. Lee. 1967. Renal homotransplantation in rats. I. Allogeneic recipients. *J. Exp. Med.* **126**:783.
11. Stobo, J. D., and W. E. Paul. 1972. Functional heterogeneity of murine lymphoid cells. II. Acquisition of mitogen responsiveness and of θ antigen during the ontogeny of thymocytes and "T" lymphocytes. *Cell. Immunol.* **4**:367.
12. Stobo, J. D., and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of T cells to phytohemagglutinin and concanavalin A as a probe for T cell subsets. *J. Immunol.* **110**:362.
13. Stobo, J. D., W. E. Paul, and C. S. Henney. 1973. Functional heterogeneity of murine lymphoid cells. IV. Allogeneic mixed lymphocyte reactivity and cytolytic activity as functions of distinct T subsets. *J. Immunol.* **110**:652