Kidney Homografts: Uptake of Fluorochrome-Labeled Tissue Extracts by Lymph Node Cells

Abstract. *Regional lymph node cells of mongrel dogs which received kidney homografts after bilateral nephrectomy take up fluorescein-isothiocyanate–labeled extracts of kidney tissue and of peripheral blood leukocytes. These fluorochrome-tissue conjugates were not taken up by cells from lymph nodes excised from the experimental dogs at the time of transplantation or from normal dogs.*

The association of cell-bound antibodies in lymphoid cells to homograft immunity has been reviewed (*1*). To our knowledge, however, the cytologic localization of antibodies in homograft recipients has not been made. The direct use of fluorochrome-labeled antigens to detect cellular antibody localization has been used successfully for insulin (*2*) and for tuberculin (*3*). We now report the use of donor tissue extracts conjugated with fluorescein isothiocyanate (FITC) to detect intracellular antibodies in the cells of the regional lymph nodes of recipients of canine kidney homografts.

Pairs of mongrel dogs were used in these kidney homograft studies. After bilateral nephrectomy of the recipient, the right kidney of the donor was transplanted to the left iliac fossa of the recipient, and the ureter was anastomosed to the bladder. The remaining donor kidney was used to prepare tissue extracts for labeling with FITC. Peripheral blood leukocytes obtained from the donor before operation were also used as a source of tissue extract for fluorochrome labeling.

Approximately 5 g of decapsulated kidney was minced with scissors and then disintegrated by ultrasonic vibration (*4*). This extract was centrifuged at 2500*g* for 10 minutes at 4°C. The protein concentration of the supernatant was determined by the biuret method and was diluted to a protein concentration of 10 mg/ml. The FITC (40 mg of protein to 1 mg of dye) was added, and, with magnetic stirring, conjugation was allowed to continue for 16 hours at 4°C. Unconjugated dye was removed by passage through a column of medium-grade G-25 Sephadex. Small quantities of the conjugate were stored at minus 20°C. Conjugates were also prepared with the extract obtained by treatment with high-frequency sound of the buffy-coat cells from blood obtained by femoral puncture of the donor dogs.

Table 1. Number of lymph node lymphocytes that showed uptake of the FITC-tissue conjugates. Two hundred cells counted per preparation.

Dog	FITC conjugate	
	Leukocyte extract	Kidney extract
JT-6	4	32
JT-7	3	19
JT-8	28	30
JT-12	32	28
BB-2	3	32
BB-3	26	58

The recipient was killed when the amount of urea nitrogen in the blood was sufficiently great and the animal appeared to be approaching a moribund state, which usually occurred by the 8th to 10th postoperative day. At autopsy, the kidney was observed for gross signs of rejection, and tissue was obtained for histologic sections. Regional lymph nodes were excised, and the lymphocytes expressed from these nodes were stained with the FITC-tissue extracts (*3*). In the examination of the preparations by fluorescent microscopy, a lymphocyte was first identified by dark-field microscopy and then examined with ultraviolet illumination to determine whether there was any uptake of the FITC-labeled extract. Uptake of the labeled extract was indicated by characteristic apple-green fluorescence of FITC in the cytoplasm of the lymphocyte. A total of 200 lymphocytes successively found were examined, and the number of positive cells was recorded.

Six pairs of dogs were studied in series No. 1. As controls, lymphocytes from regional lymph nodes excised at the time of transplantation were taken from three of the homograft recipients and stained for fluorescent microscopy with the FITC-tissue extracts. As additional controls, similar preparations were made from similar lymph nodes removed from five dogs that had laparatomies for purposes other than transplantation.

All six transplanted kidneys showed the characteristic gross signs of rejection. Sections of four of these kidneys showed histopathology consistent with rejection. Histologic sections were not done on two of the kidney homografts. The results of fluorescent microscopy of the cells from the six homograft recipients are given in Table 1. The five nontransplant dogs from whom lymph nodes were taken showed no positive cells. In two of the three transplant dogs from whom nodes were taken prior to transplantation, the cells from these nodes were negative. Four similar cells from the third dog were stained by the FITC-tissue extracts. This finding is not inconsistent with current views of antibody production.

Table 2. Number of lymph node cells that were positive when stained with FITC-tissue conjugates or with FITC-bovine serum albumin conjugate. Two hundred cells counted per preparation.

Dog	FITC conjugate		
	Leukocyte extract	Kidney extract	BSA
BB-5	4	34	0
BB-6	0	32	0
BB-7	8	26	0
BB-8	4	34	0

A second series, which consisted of four similar kidney transplants, was studied in order to rule out the possibility that the lymph node cells might be hyperactive because of the homograft and consequently might nonspecifically take up the labeled tissue antigens. In this series, in addition to testing the regional node cells with the conjugates, controls for specificity were made with bovine serum albumin (BSA) labeled with the fluorescein. None of the cells so tested were positive for the labeled BSA although the cells tested with the labeled tissue extracts (Table 2) gave results similar to those obtained in the first series. There was no uptake of the labeled tissue extracts or of the labeled BSA by cells from lymph nodes prior to transplantation.

Our results demonstrate the cytologic localization of antibodies formed during the course of a rejection of a kidney homograft. The experiments do not prove that the cellular antibodies detected are the actual instruments of homograft rejection. Nevertheless, it is possible that pursuance of this experimental approach could lead to a clarification of the nature of the antibodies or antigens, or both, concerned in homograft immunity.

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References and Notes

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5. We thank the members of Dr. T. E. Starzl's Surgical Research Unit for performing the surgery and Dr. T. A. Witten for encouragement and advice.

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Adenovirus-Associated Defective Virus Particles

Abstract. *Small, DNA-containing particles were separated from preparations of a simian adenovirus. These particles differed antigenically from the adenovirus. Replication of the particles in cell cultures was obtained only when they were inoculated simultaneously with adenoviruses. This suggests that these adenovirus-associated particles behave as defective viruses.*

Electron microscopy of simian adenovirus type 15 (SV15) has disclosed that negatively stained preparations contained a high concentration of small, uniform, virus-like particles, together with typical adenovirus particles (*1*). Our present study was undertaken to determine whether the small particles represented an unrelated virus containing SV15 or were in some manner intrinsically associated with the adenovirus.

The small particles, now tentatively referred to as adeno-associated virus (AAV), were observed first in a pooled harvest of rhesus-monkey-kidney-cell (RMK) cultures infected with SV15. These cultures had been collected and frozen when essentially complete cytopathic effect was apparent. This particular pool had been stored since 1961 at −20°C and represented a third passage in the kidney-cell cultures after the virus had been received in this laboratory (*2*). Portions (10 ml) of this and subsequent preparations were subjected to five cycles of freezing and thawing. This treatment was followed by centrifugation at low speed (12,000*g*, 15 minutes, 4°C) to separate the crude debris. The supernatant was centrifuged at high speed (60,000*g*, 90 minutes, 4°C); the pellet contained slightly purified virus particles. The reconstituted pellets were negatively stained (*3*) by phosphotungstic acid as follows. A drop of the pellet suspension was placed in contact with a carbonized, formvar-coated, electron microscope grid, blotted after 1 minute, put in contact with a drop of the stain for 4 minutes, then blotted and air dried. Two percent aqueous phosphotungstic acid was used at *p*H 1.8 through 7.4, with *p*H 4.5 giving the best results. Observations were made with a Philips EM-200 electron microscope, single or double condenser illumination being used. Photographic plates were exposed at microscope magnifications of 41,000 and 71,000.

At the lower magnification, a large number of discrete particles were observed (Fig. 1). The particles have an average diameter of 240 Å and show hexagonal profiles in many instances (arrows). Both stain-penetrated and nonpenetrated forms are apparent and correspond in appearance with known viruses, with or without nucleic acid cores (*4*). At the higher magnification, the AAV particles (Fig. 2) also show a rough surface suggesting the presence of capsomeres (*4*).

The crude suspensions of SV15 were filtered through a 50-mμ Millipore membrane to separate AAV particles from SV15. The pooled filtrates, when concentrated tenfold by high-speed centrifugation, provided a more purified particle suspension. These suspensions did not elicit adenovirus cytopathic effect after inoculation onto RMK cultures, when observed for a 14-day period. During this period, the AAV particles present in the suspensions caused no cytopathic effect in the kidney cultures, nor did the inoculated cultures yield AAV particles by electron microscopy. Likewise, cytopathic effects were not manifest within a period of 7 to 11 days after inoculation of the particles on African-green-monkey-kidney, mouse-embryo, rabbit-embryo, calf-kidney, or hamster-kidney cultures, or on a continuous cell line of KB(B) (*5*). Newborn mice and hamsters were inoculated by various routes, but showed no apparent illness 2 months after inoculation.

Purified AAV concentrates were stained for DNA or RNA content with 0.01 percent acridine orange (*6*). The yellow-green fluorescence of these preparations indicated that AAV contains double-stranded DNA (*7*). Preparations of unfixed AAV digested with pepsin and treated with deoxyribonuclease served as stain controls (*7*).

Another preparation of SV15 (*8*), hereafter referred to as SV15(M), did not contain AAV particles when examined in the electron microscope. A first passage pool, made in RMK cultures, was used to find out whether AAV could replicate in the presence of the type of virus with which it was originally found. Two-tenths milliliter of a 1 : 10 dilution of the AAV pool was added simultaneously with 0.2 ml of a 1 : 10 dilution of SV15(M) to each of two bottle cultures of RMK. Kidney-cell cultures of the same lot were similarly inoculated with SV15(M) alone or with AAV alone. Bottles were incubated at 37°C until cytopathic effects (graded 2+ or 3+) were observed in all cultures inoculated with SV15(M). Fluids were then prepared for electron microscopy. The results of this and subsequent experiments have demonstrated that AAV can replicate in RMK cultures when SV15(M) is present. The AAV particles appeared in larger numbers than adenovirus particles. The AAV-inoculated kidney-cell cultures were consistently negative for AAV as were the SV15(M)-inoculated cultures, the latter having roughly the same numbers of adenovirus particles as those inoculated with SV15 and AAV simultaneously.

A newly isolated human adenovirus type 2 preparation (*9*) was found free of AAV when examined in the electron microscope. This adenovirus, referred to now as Ad2, also had the capacity to help AAV replicate. Control cultures inoculated with either AAV alone or Ad2 alone were devoid of detectable AAV particles.

Several other DNA and RNA viruses (*10*), which themselves replicated well in RMK cultures, were similarly tested with portions of the aforementioned AAV pool with similar controls. All culture fluids were processed for observation, when suitable viral cytopathic effect was observed. All failed to induce AAV replication. In each instance a DNA or RNA virus characteristic of that inoculated was observed and photographed with the electron microscope in those cultures developing specific cytopathic effect.

A titration of one lot of purified AAV suspension was performed by inoculation of a tenfold dilution onto RMK cultures in the presence of a con-