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Renal epithelium: reversal of cytotoxic damage by addition of anti-thymocyte globulin

K. R. Clark, J. A. Kirby, N. Baker, A. L. Givan, B. K. Shenton, G. Proud, T. W. J. Lennard, and R. M. R. Taylor

Department of Surgery, The Medical School, Newcastle upon Tyne, NE2 4HH, UK

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Abstract. A novel in vitro assay of renal epithelium tight junction function was used to assess the efficacy with which rabbit anti-thymocyte globulin (ATG) blocks epithelium damage mediated by lymphokine-activated killer (LAK) cells. It was found that LAK cells lysed renal epithelial cells poorly in standard chromium-release assays but that they caused a rapid, and almost total, reduction in trans-epithelium monolayer resistance, indicating tight junction failure and, hence, loss of tissue function. LAK cell-mediated cytolysis of the sensitive K562 cell line was completely blocked in the presence of ATG at a concentration of 200 μg/ml. Addition of ATG at this concentration to damaged renal cell monolayers in the presence of LAK cells allowed the trans-monolayer resistance to recover rapidly to levels approaching the values recorded before initial addition of LAK cells. On this basis it seems likely that the rapid restoration of renal function frequently observed after appropriate "rescue" therapy during episodes of acute rejection may reflect subtle changes in tissue function rather than recovery from widespread graft cell cytolysis.

Key words: Epithelium, renal, in vitro assay – ATG rabbit, renal epithelium – LAK cells, renal epithelium

It is widely believed that cellular damage produced by cytotoxic immune effector mechanisms underlies the renal dysfunction associated with episodes of acute allograft rejection. The presence of specific cytotoxic lymphocytes within the immune cell infiltrate of organ allografts has been demonstrated in both experimental and clinical renal transplant systems. Typical experiments have shown that lymphocytes isolated from renal allograft tissue are able to lyse suspensions of donor splenic lymphoblasts [14] or donor epithelial cells [11]. However, it has also been shown that natural killer (NK) cells are able to lyse graft-derived target cell lines by antibody-dependent, cell-mediated cytotoxicity (ADCC) reactions [7] and that lymphokine-activated killer (LAK) cells, derived either from the graft or from peripheral blood, are able to lyse a range of non-HLA-identical renal cell lines [5, 6]. It is thought that similar lytic processes are involved in each of these cytotoxic interactions [12].

It is not easy to reconcile the concept that graft cell lysis is responsible for renal allograft dysfunction during rejection with the observation that appropriate "rescue" drug therapy can rapidly restore renal function [15]. It seems likely that assays of tissue function rather than of individual graft cell lysis may provide information concerning the mode of action of the drugs used in graft rescue regimes. One such assay involving in vitro measurement of the integrity of the tight junctions formed between tubular epithelial cells has recently been described [8].

In this report, the concentration of rabbit ATG required to block cytotoxic cell-mediated lysis was investigated using standard chromium-release assays. The tight junction assay was then used to monitor the rate at which tubular epithelium, which had been damaged by co-culture with cytotoxic cells, regained function after the inactivation of cytotoxic cells by addition of ATG.

Materials and methods

Monolayer culture of kidney cells

A continuously proliferating sub-clone of cultured human embryonic kidney cells (F4000c2; Flow Laboratories, Hertfordshire, UK) was routinely propagated on 25 cm^2 flasks (Falcon; Becton Dickinson, Oxfordshire, UK) in complete Eagle's minimum essential medium (Northumbria Biologicals, Northumberland, UK) containing 10% (v/v) heat-inactivated foetal calf serum, HEPES buffer (pH 7.3; 10×10^{-3}M), ampicillin (100 μg/ml) and streptomycin (100 μg/ml), which was additionally supplemented with sodium pyruvate (Sigma Chemicals, Dorset, UK) and non-essential amino acids (Sigma). Confluent cultures were split in the ratio 1:3, as necessary, using trypsin-EDTA (Northumbria Biologicals) to release the adherent cells.

Cell monolayers were prepared on Anocell 10 tissue culture inserts (Anotec Separations, Oxfordshire, UK) by seeding 5×10^5 cells onto each 50 mm^2 membrane and incubating the culture plate inserts

Offprint request to: K. R. Clark

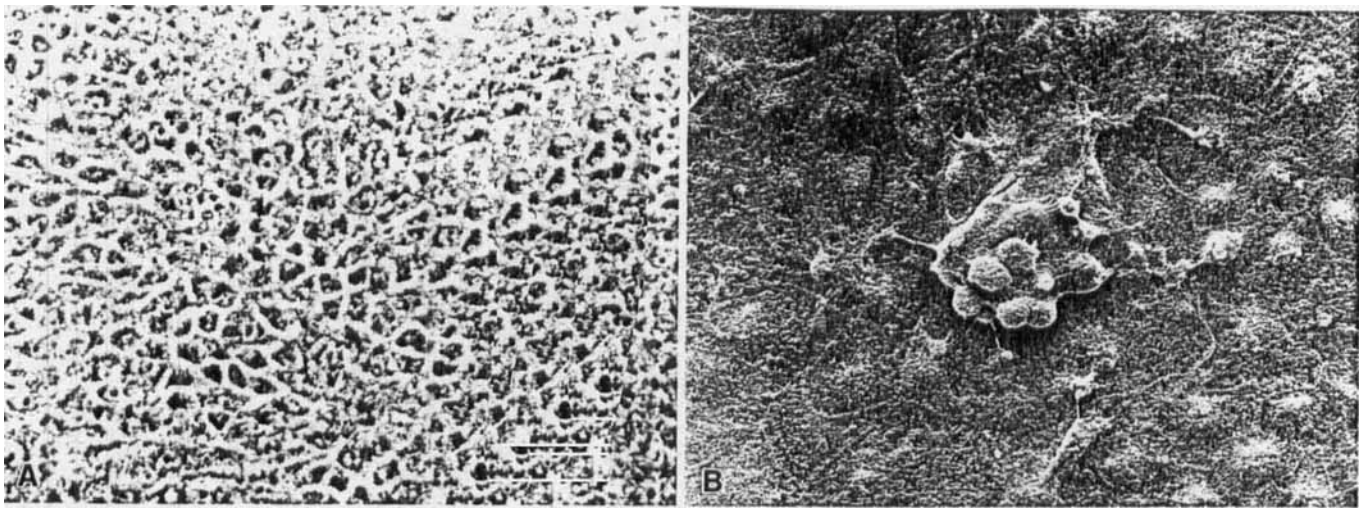

Fig. 1 A, B. Monolayer of F4000c2 cells propagated on porous membrane: **A** phase contrast micrograph through Anocell culture insert (× 300); **B** scanning electron micrograph (× 900)

in 24-well tissue culture plates (Costar Plastics; Northumbria Biologicals). The development of functionally defined monolayer integrity was monitored by recording daily trans-monolayer electrical resistance values; all the resistance measurements were corrected for background resistance of the filter matrix. The resistance values were measured using a combined alternating current source and milli-voltmeter connected to two "chopstick" Ag/AgCl electrodes (Millicell-ERS; Millipore, Hertfordshire, UK). One of the electrodes was placed above the cell monolayer in the culture insert and the other was placed in the medium surrounding the insert; the distance between the electrodes was held constant by means of a small clamp.

Preparation of cytotoxic effector cells

LAK cells were prepared using a method described previously [5]. Briefly, heparinised peripheral blood taken from normal volunteers was diluted 1:1 with RPMI 1640 (Northumbria Biologicals) and centrifuged for 25 min at 400 *g* over a ficoll-metrizoate (Lymphoprep; Nycomed, W. Midlands, UK) density gradient. The interfacial band of mononuclear cells was recovered and washed twice by centrifugation. The final pellet was resuspended in complete RPMI 1640 medium containing 10% (v/v) heat-inactivated foetal calf serum, HEPES buffer (pH 7.3; 10×10^{-3}M), ampicillin (100 µg/ml) and streptomycin (100 µg/ml) at a concentration of 1×10^6 cells/ml, and 10-ml aliquots were incubated for 5 days in upright 25 cm^2 flasks in the presence of recombinant IL-2 (Boehringer Mannheim, FRG; East Sussex, UK) at 25 U/ml. After this time the LAK cells were washed once by centrifugation and resuspended in complete culture medium.

Dysfunction of kidney cell monolayers

Dysfunction of kidney cell monolayers was monitored by measuring changes in the trans-monolayer resistance of cell monolayers that had previously developed a stable trans-monolayer resistance value [8].

Assays of dysfunction mediated by LAK cells were performed by replacing medium above the monolayer with a suspension of 2×10^6 LAK cells in complete culture medium. After addition of LAK cells, the trans-monolayer resistance was measured at intervals up to 540 min. After recording a significant LAK cell-mediated drop in trans-monolayer resistance, monolayers were treated with 200 µg/ml of either rabbit ATG (Institut Mérieux, Lyon, France) or control rabbit IgG (Sigma).

Cellular cytotoxicity assays

Approximately 1×10^6 of F4000c2 or K562 cells (human erythroleukemia line; ECACC, Porton Down, UK) were resuspended in 100 µl of RPMI 1640 medium containing 200 µCi of $Na_2{}^{51}CrO_4$ (600 mCi/mg per Cr; CJS-4; Amersham International) for 90 min. The cells were then washed three times by centrifugation in complete medium and adjusted to 2.5×10^4 cells/ml. One hundred microliters of the labelled F4000c2 target cells was mixed with 100 µl of effector LAK cells at effector-to-target ratios of 50:1, 25:1, 12.5:1, 6.25:1 and 3.13:1 in the wells of round-bottom, 96-well plates. Assays were also performed using labelled K562 cells and LAK cells at a constant effector-to-target ratio of 50:1 in the presence of varying doses of either ATG or rabbit IgG (between 0 and 250 µg/ml). Maximal release of ^{51}Cr was determined after two freeze-thaw cycles of the target cells, and spontaneous release was measured after incubation of the target cells in the absence of effector cells. After incubation for 4 h, the plates were centrifuged at 200 *g* for 5 min to pellet the cells, and 100 µl of medium was recovered from each well for gamma spectrometry (LKB-Wallac Clinigamma 1272; Pharmacia-LKB, Milton Keynes. UK). The percentage specific release of ^{51}Cr due to the action of cytotoxic effector cells was calculated using the following equation [1]:

$$\text{Percentage specific } {}^{51}\text{Cr release} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

Results

The F4000c2 cells showed a polygonal morphology in culture and formed microscopically confluent monolayers rapidly after seeding onto untreated culture insert membranes (Fig. 1). Before seeding with cells, the membranes showed an electrical resistance of 203 ± 5Ω (mean ± SD; $n = 5$) when incubated in complete Eagle's minimal essential medium. After seeding the membranes with F4000c2 cells, the resistance attributable to these cells increased

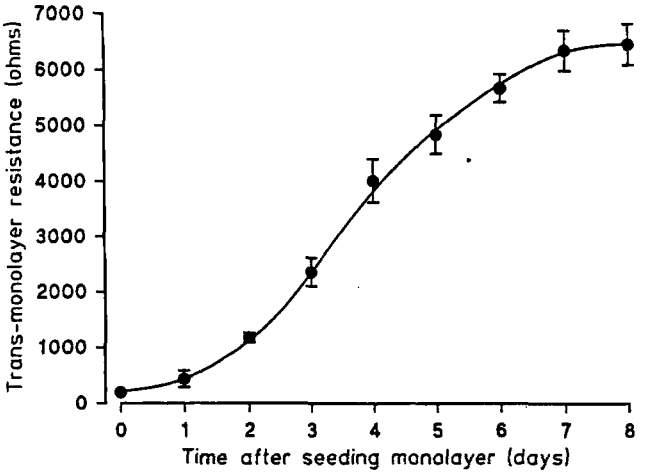

Fig. 2. Increase in trans-monolayer electrical resistance of F4000c2 cells seeded at 5×10^5 cells per membrane. The *points* represent the mean resistance of five monolayers and the error *bars* show the standard deviation

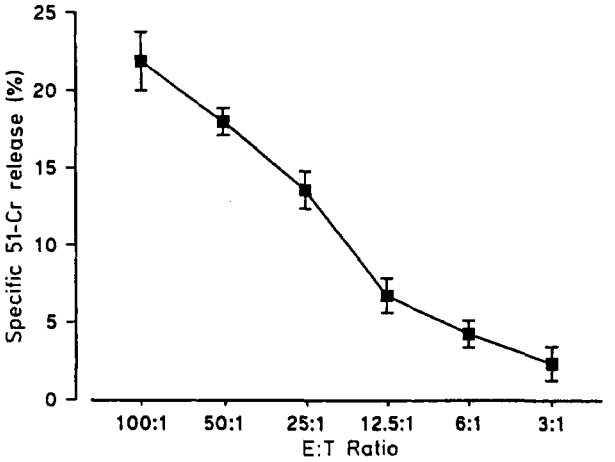

Fig. 3. LAK cell-mediated cytotoxicity of F4000c2 cells in a 4-h assay. The *points* represent the mean of five replicates and the error *bars* show the standard deviation

rapidly (Fig. 2) and reached a stable value of $6448 \pm 368\Omega$ (mean ± SD; $n = 5$) after a period of 8 days. It was found that as the pass number of the cells increased, the maximum trans-monolayer resistance produced by the cells decreased, as did the period required to develop a stable maximum trans-monolayer resistance value. Recovery of adherent cells by trypsinisation of three confluent monolayers gave a mean yield of 4.1×10^5 F4000c2 cells per Anocell culture insert.

The results of a representative chromium-release assay performed using suspended ^{51}Cr-labelled F4000c2 cells as targets and LAK cells as effectors are shown in Fig. 3. These data show that at the highest effector-to-target cell ratio tested, the specific ^{51}Cr-release did not exceed 25%. In order to determine the efficacy with which ATG blocks LAK cell-mediated cytolysis, it was decided that the K562 cell line, which is readily lysed by LAK cells, should be used as cytotoxicity assay targets in order to increase the sensitivity of the system. The results of chromium-release assays performed between K562 target cells and LAK cell effectors in the presence of varying doses of ATG or control rabbit IgG are shown in Fig. 4. These results show that all the K562 cells were lysed by LAK cells at an effector-to-target cell ratio of 50:1 within a 4-h assay period. However, addition of increasing doses of ATG at the start of the experiment depressed target cell lysis; this depression was complete in the presence of ATG at concentrations of between 125 and 250 µg/ml. A similar reduction in cytolysis was not observed using comparable doses of control rabbit IgG. Additional control experiments showed that neither ATG nor rabbit IgG were cytotoxic at any of the concentrations used in the assay. Further experiments (not shown) demonstrated that ATG at a dose of 200 µg/ml was sufficient to block LAK cell-mediated cytolysis of F4000c2 at an effector-to-target cell ratio of 50:1. On the basis of these results, ATG was used at a concentration of 200 µg/ml in all subsequent experiments.

The representative results in Fig. 5 show the effect of adding 2×10^6 LAK cells to high-resistance F4000c2 monolayers on trans-monolayer resistance. The data show a rapid decrease in trans-monolayer resistance within the first 30 min after addition of the LAK cells; after 90 min the trans-monolayer resistance stabilised at between 10% and 20% of the initial value. Addition of fewer than 2×10^6 LAK cells per monolayer produced smaller and less rapid decreases in resistance. However, increasing the number of cells beyond 2×10^6 did not yield a concomitant increase in the rate or extent of resistance decay. Simultaneous addition of 2×10^6 LAK cells and ATG at 200 µg/ml produced no detectable reduction in trans-monolayer resistance (results not shown). Furthermore, addition of this dose of ATG 90 min after addition of the LAK cells (Fig. 5) rapidly reversed the decrease in trans-monolayer resistance and allowed the resistance to recover to over 4000Ω within a period of 450 min. At this time the trans-monolayer resistance appeared to plateau at 70% of the initial resistance. Addition of control rabbit

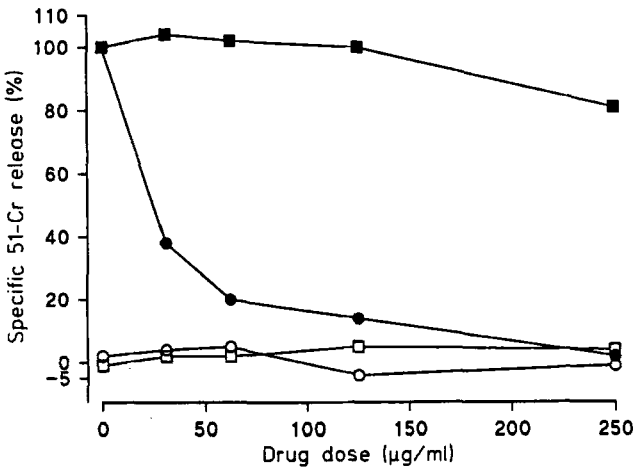

Fig. 4. LAK cell-mediated cytotoxicity of K562 cells after 4 h at an effector-to-target cell ratio of 50:1. ATG was titrated into one series of experiments (●) and rabbit IgG was added to a second series (■). Background cytotoxicity of ATG (○) and of rabbit IgG (□) was tested in the absence of LAK cells. The *points* represent the mean of triplicate determinations

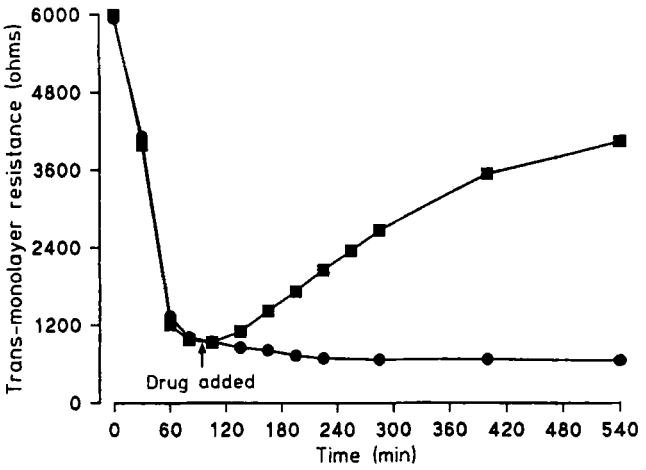

Fig. 5. Representative changes in trans-monolayer electrical resistance after the addition of LAK cells. After the LAK cells had been present for 90 min, 200 μg/ml ATG was added to one monolayer (■) and rabbit IgG was added to a second monolayer (●)

IgG after 90 min did not produce a significant recovery of trans-monolayer resistance during the period of the experiment.

Discussion

The continuous line of kidney cells used in this series of experiments was identified as epithelial in character on the basis of the "cobblestone" morphology when cultured to confluency on porous membranes (Fig. 1). This appearance has been described previously for primary cultures of renal tubular epithelial cells [16]. Furthermore, the development of a high trans-monolayer electrical resistance after seeding the cells onto membranes (Fig. 2) is indicative of the formation of tight junctions between the cells [2, 4]. Such tight junctions are characteristic of functional, mammalian, renal, epithelial cell lines, such as the low passage number "strain 1" MDCK cell line [13]. The maximal resistance produced during this series of experiments was approximately three times greater than that previously reported for F4000c2 cells [8]. It is likely that this change in resistance is related to the pass number of the cells; it has been previously reported that the T84 line of gut epithelial cells develops a maximal resistance that decreases with increasing pass number [9].

The results in Fig. 3 show that F4000c2 are lysed poorly by LAK cells, with fewer than 25% of the target cells being lysed by a 100-fold excess of effector cells in a 4-h assay. By contrast, K562 cells were completely lysed by a 50-fold excess of LAK cells in a similar period of time (Fig. 4). The results in Fig. 4 show that ATG was effective at blocking LAK cell-mediated cytolysis of K562 cells when added at the start of the cytotoxicity assay; a drug concentration of 62.5 μg/ml reduced target cell lysis to less than 20% whilst complete protection against lysis was achieved at a concentration of between 125 and 250 μg/ml. The ability of ATG to prevent lymphoid cell-mediated damage in the absence of complement is probably due either to blockade or to modulation of the cell surface receptors involved in immune cell binding and activation [3]. The concentration of ATG necessary to prevent LAK cell-mediated cytolysis was greater than the plasma concentration (20–101 μg/ml) used to treat renal allograft rejection [10]. This may be explained by the presence of complement in plasma, enhancing the efficacy of the drug by facilitating immune cell lysis.

The results in Fig. 5 show that LAK cells added to F4000c2 monolayers at an approximate effector-to-target cell ratio of 5:1 are sufficient to cause a maximal 80%–90% drop in trans-monolayer electrical resistance and, hence, in tight junction function within a period of 90 min. These resistance results contrast with the cytotoxicity assay results (Fig. 3), in which an effector-to-target cell ratio of 6:1 produces a barely detectable release of ^{51}Cr from suspended F4000c2 cells. The failure of an increased effector-to-target ratio to enhance the rate of resistance decay may be due to cellular steric hindrance effects on the monolayer or to overloading the low-volume culture system with cytotoxic cells. It has been demonstrated previously that addition of freshly isolated peripheral blood mononuclear cells causes no change in trans-monolayer resistance [8].

Addition of ATG to damaged monolayers that had been incubated with LAK cells for 90 min resulted in rapid recovery of trans-monolayer resistance. Indeed, the resistance was restored to 70% of the initial value within 450 min of addition of the drug. This action of ATG was specific, as control rabbit IgG was not able to reverse the deterioration in trans-monolayer electrical resistance. In view of the time required to generate high-resistance monolayers (Fig. 2), it is unlikely that restoration of high resistance after the addition of ATG to LAK cell-damaged monolayers is caused by the F4000c2 cells dividing to fill "holes" in the monolayer left by lysed cells. It is more likely that LAK cells, at the low effector-to-target cell ratio employed, mediate little lysis of F4000c2 cells in the monolayer but that these cytotoxic cells are somehow able to perturb intercellular tight junctions. Functional inactivation of these LAK cells by treatment with ATG may then allow rapid restoration of the tight junctions with an associated increase in trans-monolayer electrical resistance.

Although there is only limited evidence that non-specific cytotoxic effector cells, such as LAK cells, play a significant role in vivo during renal allograft rejection, it is apparent that both LAK cells and specific lymphocytes, which do appear to be active during rejection, mediate cell lysis by similar mechanisms. Hence, LAK cells can be used in vitro to model cellular damage that may be inflicted in vivo on renal epithelium by cytotoxic. Work is currently in progress to define the mechanism by which cytotoxic effector cells mediate dysfunction of trans-monolayer resistance.

In conclusion, it has been demonstrated that LAK cells are able to disrupt the function of renal epithelial cell monolayers but that subsequent inactivation of these cytotoxic cells by treatment with ATG results in rapid restoration of intercellular tight junction integrity and monolayer function. Processes similar to this may be responsible for the rapid recovery of graft function observed after rescue therapy of acutely rejecting renal allografts.

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