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Experimental strategies for the study of cellular immunity in renal disease

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Over the last several years there has been a renewed interest in basic cellular mechanisms of immunologic renal injury. This renaissance has broadened and challenged our traditional views of what constitutes a critical mechanism of renal damage by assigning cell–mediated effector processes their proper place alongside those of immune deposits and kidney–specific antibodies. This new awareness of cellular mechanisms has also stimulated research into the cell–mediated regulatory circuits controlling both antibody and T cell effector events and, with the enormous growth in immunogenetics and molecular biology, now provides new opportunities for understanding the basis of disease susceptibility at a molecular level [1]. With the current capability to develop monoclonal cell lines and T cell hybridomas, the availability of genetic recombinant rodent species, as well as antibodies to a wide assortment of cell phenotypic and histocompatibility gene products, a whole new area of immunosemiotics has evolved within the field of basic immunology. Immunosemiotics is the study of interactive signals that modulate the response of immune cells, and is concerned with issues involving cell receptor–ligand analyses, soluble factor characterizations, and the genetic rules of cell–mediated communication which govern self and non-self recognition [2]. It is now increasingly possible to apply this body of working knowledge to problems in renal immunology. For the purposes of this current discussion, it is our intention to review the basic premise of genetic restriction, the routine methods of cellular immunology, and some general strategies for using T cell–mediated immunity to answer questions relevant to renal immunopathology.

Genetic restriction of T lymphocytes

Of the many testable gene products those of the major histocompatibility complex (MHC) have been most convincingly demonstrated to have a role in the genetic restriction of immune responses by T lymphocytes [3]. The MHC, among other things, codes for both Class I and Class II molecules [4, 5]. These determinants are well represented on the surface of most lymphocytes, and at a structural level they consist of two chains with a transmembrane tail piece.

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It has become apparent over the past decade that most helper/inducer or cytotoxic T cells do not directly bind antigen [3, 6–9], unlike suppressor T cells [10, 11] and B lymphocytes [12]. Instead, helper and effector cells recognize nominal antigen only when it is presented in the context of a particular gene product from the MHC [6, 9]. This is most readily envisioned by thinking of the T cell receptor as having either one combining site which recognizes a complex between nominal antigen and MHC molecule, or having distinctive dual specificities, one for nominal antigen and one for the MHC [7, 8, 13]. Such phenomena were initially revealed in studies which showed that T cells educated to antigen in the presence of syngeneic MHC determinants were ineffective in responding to the same antigen in an allogeneic format [14–16]. A requirement for functional homology between MHC molecules of the responding T cell and the MHC of the antigen–presenting cell becomes a true genetic restriction when it is consistent for all polymorphisms at a given locus of interest. With exceptions, many helper/inducer T cells are restricted by MHC Class II molecules [3], while cytotoxic T cells are typically restricted by Class I determinants [9]. T cell receptor restrictions are also seemingly facilitated by associative–recognition molecules that modulate low avidity cell–cell interactions [17, 18]. Class II interactions can be facilitated by cell–surface L3T4 determinants in mice and Leu 3/T4 determinants in humans; Class I interactions can be modulated by Lyt-2 determinants in mice and Leu 2/T8 determinants in man [17]. While critical genetic restrictions involve MHC interactions, there are also other gene loci with potential restrictive effects. These loci have been best defined in mice where recombinant events can be mapped with some precision, and include Igh-V [19], IgT-C [20], and I-J regions [21]. These latter restriction sites cannot replace the implicit requirements for MHC gene products, but probably act in a complementary or co-interactional manner.

Methods

Experimental questions of cellular immunity in renal disease often require the implementation of many research techniques. These methods have evolved from the field of basic immunology and, for the most part, are easily adaptable to an analysis of the nephritogenic immune response.

Cell preparation

The lymphoid system may be divided into primary lymphoid organs (the bone marrow, bursa–equivalent, and thymus) where lymphocyte maturation and education occurs, and secondary organs (spleen and lymph nodes) where antigen-presentation generally initiates an immune response. Many of these latter lymphocytes also circulate with distinct homing patterns [22].

The lymphoid system contains B cells which function in the humoral immune response and T cells which are responsible for cell–mediated immunity. Distinct T cell subsets serve as inducer or helper cells, suppressor cells, or effector cells for cytotoxicity, delayed-type hypersensitivity, or cutaneous sensitivity. In addition to these cells, the lymphoid system also contains dendritic cells and macrophages, which present antigen as well as natural killer cells. Natural killer cells are pre-programmed to directly lyse selected target cells [23]. These large granular lymphocytes are not T cells, B cells or macrophages, but they can express a receptor for the Fc portion of IgG. Their lineage is not fully established.

Very often a specific cell population needs to be isolated to ask a relevant question about a selected immune response. The spleen and lymph nodes are easily retrieved from sacrificed animals and are the usual source of functionally mature lymphoid cells. About two thirds of splenic lymphocytes are B cells whereas about two thirds of lymphocytes in peripheral nodes are T cells, depending on the strain [24]. To harvest these cells, the lymphoid organ is minced, gently teased apart, or pushed through a wire mesh screen. Viability and cell counting is then determined by trypan blue dye exclusion. Counting should be completed within three minutes of adding the dye and the media should not contain protein. Peripheral blood can be enriched for lymphocytes with Ficoll–hypaque gradients [25].

Positive and negative cell selection

Harvested lymphocytes can be separated into subpopulations with polyclonal or monoclonal antibodies to discriminating cell–surface determinants. The characterization of these determinants in man [26–28], mice [17, 29–33], and rats [34] has been reviewed elsewhere. The separation of functionally distinct subpopulations can be accomplished by means of positive selection in which cells of appropriate phenotype are enriched, or by negative selection in which selected populations are depleted by antibody and complement.

Negative selection in vitro can often be performed as a one–step procedure (adding the antibody and complement at the same time) with many monoclonal antibodies, whereas depletions with polyclonal antisera are usually done in two–steps [35, 36]. The antibody has to be complement–fixing and the source of complement is critically important. The optimal dilution of antibody and complement per cell number must be independently established and validated by viable cell counts of anticipated remaining live cells or by fluorescent labelling studies. If large numbers of cells are processed this way it is often useful to add a little DNAase (10 to 15 μg/ml) to obviate clumping. In vivo cell depletions are also possible with complement–fixing antibodies [37, 38], although IgM antibodies do not penetrate peripheral lymphoid organs very well and anti-antibody reactions develop quickly.

Positive selection for enrichment of macrophages can be performed by incubations on culture dishes at 37°C [39]. Macrophages can be removed from the dishes by temperature shift, EDTA, or lidocaine. Lymphocyte populations can be separated into T and B cell components with nylon wool columns at 37°C [40]. More recently it has also become feasible to separate large number of lymphocytes into T and B cells, or into T cell subpopulations using antibody–coated panning dishes [41, 42]. This is particularly useful when a cell population needs to be enriched for definition, or if the discriminating antibodies are not complement–fixing. Polystyrene petri dishes can be pre-treated with affinity–purified antibody of defined specificity, and after a brief incubation, the non-adherent cells can be removed. The remaining adherent cells are released by temperature shift and pipetting, although if the antibody is of a too high affinity, the cells cannot be dislodged. This problem can be largely avoided by using optimal concentrations of affinity–purified anti-antibody to coat the dishes and pre-incubating the cells with the cell–surface antibody of interest before adding the cell mixture to the panning dish. This indirect method will usually allow cells to release after the non-adherent population has been gently washed away. The efficiency of separation must be established in each laboratory at defined temperatures for a given cell number relative to exposed surface area coated with optimal antibody. Finally, it is also possible to perform accurate cell separations on a fluorescence–activated cell sorter [43]. Such separations can be useful for cell cloning and enrichment, although the efficiency is poor and impractical when large cell numbers are needed.

Long term culture of T lymphocytes

Characterization of the complex biochemical and cellular interactions that occur in the immune system is often exceedingly difficult using in vivo systems. Fortunately, over the last ten years, investigators have developed techniques for cloning or immortalizing T cells in vitro such that their biochemical properties can be more easily evaluated. Crucial to continuous T cell growth in culture was the discovery of T cell growth factor, or IL-2, from the supernatants of lectin–stimulated lymphocytes [44]. IL-2 enabled the first immortalized growth of a cytotoxic T cell line [45] and is now employed routinely in many culture systems.

IL-2 can be prepared by culturing mouse or rat splenocytes in media containing mitogen and then using the supernatant directly as 10 to 25% of fresh T cell culture media. The IL-2 can be further purified by vacuum dialysis, gel filtration, ion exchange chromatography, and isoelectric focusing [46]. While IL-2 production by non-transformed cells is relatively low, there are human leukemia [47] and mouse lymphoma [48] cell lines that produce 1,000 to 10,000 times the IL-2 of non-transformed lines. Even these lines, however, still require Con A or PHA for stimulation which can contaminate T cell growth media and act as a non-specific mitogen. Mitogen can be largely removed as an issue by pulsing the splenocytes for two hr, washing it away, and letting the cells complete a culture interval of 48 h [49]. There also is a gibbon cell line, MLA-144, available which spontaneously secretes large amounts of IL-2, thereby obviating the need to remove mitogenic contaminants [50]. To alleviate concerns of unknown factors contained in supernatants, one can utilize commercially available recombinant IL-2

[51]. At some time during the use of a particular type of IL-2, it is prudent to bioassay activity by measuring thymidine incorporation of the IL-2 dependent lines CTLL [52] or HT2 [53].

There are two general methods for cloning T cells, soft agar and limiting dilution, each of which has relative strengths and weaknesses. The soft agar technique involves suspending recently (24 hr) stimulated T cells at various concentrations in 0.5% agar with subsequent layering of this suspension over another layer of several million syngeneic irradiated feeder splenocytes. After a few days in culture at 37°C, colonies can be found in the semi-solid agar and picked with a drawn-out pasteur pipette and expanded in successfully larger culture dishes [54, 55]. The technique offers some security that clones come from monoclonal colonies. Limiting dilution performed in microtiter plates, on the other hand, offers a simpler system to assess clonotypic heterogeneity, but the monoclonality of lines so produced is really more of a statistical assumption than the physical evidence suggested by the soft agar technique.

Limiting dilution for cloning of alloreactive cells has been done as follows [56–59]: T cells from a previous mixed-lymphocyte reaction are stimulated with excess allogeneic, irradiated feeders for one to two days, and then plated at 20, 10, 5 and 1 cells/well with one million fresh allogeneic feeders in 0.2 ml microtiter plates with IL-2. A week later, wells positive for growth are expanded into 2 ml wells with fresh feeders in IL-2. Subsequent subcloning is done at 0.3 cells/well, and after several such procedures, monoclonality is assumed. This technique has been used to generate helper/inducer and cytotoxic T cells. When one tests for proliferation or cytotoxicity, assays should be done in the presence and absence of IL-2, as some clones may not proliferate specifically in its absence.

Often one is interested in nominal antigen-reactive T cells which can also be cloned from animal lymphoid organs [60] or human blood [61]. Nominal or synthetic antigen is adjuvant is injected into the donor, and after a week or so, lymphoid cells are harvested and passed over a nylon wool column to remove B cells and monocytes. The non-adherent T cells are cultured at 1×10^6/cc with equal numbers of fresh irradiated syngeneic feeders, and antigen at optimal dilution. After one or two culture periods, IL-2 is introduced into the system [60, 62]. This technique has been used to generate cell lines which can passively transfer experimental allergic encephalomyelitis in rats [59] and mice [63], and also to generate a helper/inducer cell which is capable of inducing anti-TBM nephritis in susceptible strains of mice [64]. It is possible to test these cells in vitro for proliferation to antigen and for functional effect as well as for fine specificity and genetic restriction. Suppressor cell clones can also be generated, although it often requires selective removal of other classes of immune T cells or enrichment of the suppressor lymphocyte, and a sensitive assay for suppression [65–68]. An important methodological variable may be the dose of antigen used to define a supressive event [69].

Maintenance of T cell clones in long-term culture requires continual restimulation, co-culture with feeders, and careful monitoring of culture conditions. If one wishes to purify helper or suppressor factors from these cultures, it is frequently difficult to grow enough clones to yield the quantities needed. Accordingly, following the protocols used for somatic fusion of antibody-secreting cells with myeloma cells [70], investigators developed methods for immortalizing T cells by fusing specific immune T cells with T cell lymphomas [71, 72]. Generally, one requires a T lymphoma line with a metabolic defect such that unfused cells will not grow in a defined selection medium. The viability and growth phase of the starting cell populations, the source of polyethylene glycol, and the length of the mixture reaction are all critically important.

With T cell hybridoma technology, a variety of helper [73, 74] and suppressor factors [75–76] have been purified for analysis. The hybridomas can produce ascites in appropriate F_1 hybrids or nude rodents. This material provides a rich source of lymphokines which can be used in passive transfer studies where the cells cannot. Over time, fusion lines can loose chromosomes [53, 57, 77] and stop growing so that repetitive subcloning is usually mandatory.

Preparation of helper and suppressor T cells and their soluble factors

The helper T cell repertoire facilitates and augments complex antigen-reactive immune responses. These cells induce and direct the differentiation of cytotoxic lymphocytes, T cell-mediated delayed-type hypersensitivity, and B cell maturation. In fact, the effector cell response to most complex antigens requires inducer T cells to provide cognate or polyclonal help. Cognate help is MHC-restricted [64, 78, 79] and mediated by a specific group of helper/inducer cells which form an interactive relationship with other differentiating T and B cells [80, 81].

Helper cells capable of releasing soluble helper factors can be induced in several ways. Such cells may be harvested from the spleens of lethally irradiated mice who receive of thymocyte cell-transfers and are immunized with antigen [82]. Such cells, when restimulated in vitro, release an antigen-specific helper factor [83]; and cell extracts are also a source of this soluble material [84]. Alternatively, T cells of antigen-primed mice in vitro have been shown to produce helper factors [85, 86], and their established functional effects can be carried as cell lines [65–87, 88]. T helper/inducer cells in murine interstitial nephritis, as long-term cultures, are capable of inducing effector T cells in vitro which subsequently produce disease on adoptive transfer [64]. Such helper cells also produce a soluble factor which can mediate this inductive effect (unpublished observation). Finally, helper cell hybridomas have been generated as a source of soluble factors, although the amounts generated seem to be less than those produced by suppressor cell fusions [89, 90].

In the last several years, it has also become apparent that suppressor mechanisms play a major role in the modulation of both humoral and cell-mediated immune responses [1, 91, 92]. Such suppression may be due to the formation of anti-idiotypic antibodies or suppressor cell networks. Suppressor cell networks typically involve several sets of sequentially activated, suppressor T cells which have complementary specificities, operate under genetic restrictions, and secrete soluble suppressor factors which mediate complex interactive functions [1, 6, 12, 76, 93–100]. Suppressor networks have been demonstrated in a variety of renal diseases, either as part of the natural evolution of a nephritogenic immune response [101, 109], or as investigator-induced therapeutic interventions [110–114]. Suppressor cells may be induced in vivo with ligand-coupled lymphocytes [92, 115], high dose antigen-priming without adju-

vant [116], or with antigen in incomplete Freund's adjuvant [112, 113]. Suppressor cells can also be induced in in vitro culture systems [117]. Once induced, these cells can be harvested and isolated in several ways. Many suppressor T cells are antigen–binding and will do so without antigen processing and presentation, using antigen–coated plates [10, 11]. Alternatively, cells may be incubated with antibodies to cell–surface determinants (such as αI-J, αLyt-2, αL3T4, αT4, or αT8) and selected by cell sorter or indirect panning. Once selected, like helper cells, they can be characterized for phenotype and genetic restriction, and can now be grown as continuous cell lines [65–69], or as hybridomas after fusion [96, 118]. It is also evident that suppressor T cells secrete soluble suppressor factor [90, 119, 120]. Different methods of factor preparation have led to some conflicting results regarding their properties [121, 122]. To some degree these discrepancies may be explained by subpopulation contamination [123], and by the fact that different suppressor factors may operate at different points in the effector response [124, 125]. Like the helper cell factors, suppressor molecules can also be subjected to a variety of bioimmunochemical analyses [90, 118–121].

In vitro correlates of T cell–mediated immunity

As lymphocyte activation and proliferation represent the sine qua non of the antigen–reactive immune response, in vitro assays of proliferation are frequently employed in the study of immune–mediated disease. Lymphocyte proliferation is typically a cell interaction event. Accessory cells (macrophages and dendritic cells) activate antigen–specific T helper/inducer lymphocytes through their capacity to process and present antigen in association with class II MHC gene products [126–128] and to secrete IL-1 [129]. IL-1 subsequently facilitates IL-2 production by inducer T cells [130–132] which, in turn, stimulates the proliferation of other antigen–reactive T cells [133–137]. Either IL-1 or IL-2 can replace the requirement for accessory cells in some culture systems [138]. In addition to its facilitative effects on antigen–specific T cells, IL-2 also enhances natural killer cell activity [139]. Other T cell lymphokines stimulate B cell differentiation [140, 141] or macrophage expression of MHC antigens [142–143]. This latter effect may amplify ongoing proliferative events.

How in vitro measurements of T cell proliferation correlate with in vivo expression of immune responsiveness must be independently established for each system. When stable T cell lines are used for analysis, it is somewhat easier to analyze proliferation against measured function [64]. They are, however, not the same, nor is either an easily substituted measure for the other [144]. The most convenient and widely used assay for lymphocyte proliferation in response to antigen, mitogen, or allogeneic cells is the measured incorporation of tritiated thymidine (^{3}HTdR) [145–148].

Problems in interpretation can be minimized if results are regarded simply as rough approximations of changes in DNA synthesis [149]. Collected data can be expressed as raw counts: a delta cpm, or as a stimulation index or ratio of stimulated versus resting cells. Appropriate controls for proliferation assays consist of cells pulsed with ^{3}HTdR in the absence of antigen as well as the use of "irrelevant" antigens to determine the specificity of the measured response. Proliferation assays can also be used to address the genetic restriction of lymphocyte activation as well as the role of associative–recognition molecules [64]. In the case of long term T cell lines, these events can be correlated with the emergence of predictable function.

T-B cells interactions can also be analyzed in vitro by measuring hemolytic plaques which are the result of a small amount of lytic antibody secreted in the vicinity of a single B lymphocyte [150]. While there are a variety of methods for plaquing, typically immune lymphocytes and ligand–coated red blood cells are mixed in a thin layer of agar. After incubation, the addition of complement permits the lysis of IgM antibody–coated indicator cells (direct plaques), or the difference between complement alone and anti-IgG, IgA, or IgE and complement (indirect plaques) when secretion by other classes of antibody are of interest [151]. IgM plaques can also be inhibited by the presence of 2-ME or DTT, making the detection of IgG plaques less cumbersome [152]. A variety of haptens and proteins can be linked to indicator red blood cells using covalent–coupling reactions [153]. Red blood cells can also be coated with protein A to measure polyclonal antibody responses to B cell mitogens or the presence of anti-idiotypic antibodies in antigen–activated systems [154]. These reverse–hemolytic plaque assays are very powerful tools in the analysis of the B cell repertoire. Other investigators have also used radioimmunoassay to detect secreted antibody with good effect [155–158]. These B cell assays have been employed in the evaluation of T-B cell collaborations using a variety of strategies [159, 160], and have been recently used to study the B cell response in renal disease [103, 106, 158]. Admixing helper/inducer T cells and B cells with putative suppressor T cells also allows for the analysis of interactive regulatory events [66, 103, 161].

The ability of immune lymphocytes to specifically destroy target cells was first demonstrated by Govaerts in 1960 with thoracic duct lymphocytes from dogs receiving renal allografts which were cytotoxic to kidney cells of the allograft donor [162]. In the decade following this discovery, a variety of methods were developed to assay cytotoxicity. These included microscopic detection of damage to target cell monolayers, quantitative counting of viable target cells following incubation with effector cells, and inhibition of target cell colony formation by effector cells [163]. These techniques have been largely superceded in the last fifteen years by methods utilizing the release of isotopes from labeled target cells. Chromium-51 (which is not reutilized) has emerged as the most sensitive and reliable radioactive marker for assessing cytotoxicity. The standard chromium release assay was described by Brunner et al in 1968 [164] and has undergone multiple minor modifications in subsequent years [165–167]. In typical ^{51}Cr release assays, viable effector lymphoid cells, obtained from immune blood or peripheral lymphoid organs, are admixed with ^{51}Cr labeled target cells in varying effector/target ratios. Following three to six hours of incubation at 37°C, the resultant supernatants are counted for released ^{51}Cr. With high rates of spontaneous isotope release this assay loses sensitivity and reproducibility. Although modifications of the standard ^{51}Cr release assay have been shown to correlate well with the functional ability of cytotoxic cells to inhibit target cell clone formation [168], this assay is probably inadequate to address questions of relative numbers of cytotoxic cells in different populations, as it does

not distinguish quantity from lytic efficiency [169]. Such issues are more appropriately addressed using limiting dilution detection of cytotoxic T cell precursors [170]. The role of more recently described colormetric cytotoxicity assays remains to be defined [171].

Proper interpretation of cytotoxicity assays depends on the inclusion of appropriate experimental control groups and characterization of the effector and target cell populations. Effector lymphocytes from non-immunized animals establishes the background cytotoxicity for a given strain; the specificity of cytotoxicity is established with target cells expressing irrelevant antigen. Specificity, as well as the presence of different populations of cytotoxic cells, can also be established by cold target inhibition [172].

Ascribing cytotoxic effector function to a particular subpopulation of immune lymphocytes requires homogeneous cell populations. In recent years, the issue of contaminating macrophages and polymorphonuclear leukocytes in lymphoid populations has been largely obviated by the use of monoclonal antibodies, cell separation procedures, and T cell clones. The visibility of target cell associated Class I MHC molecules is another variable which may differ markedly between in vivo and in vitro settings. In studying the role of cytotoxic T cells in immune–mediated kidney disease, choice of target cells is critical if one is to ascribe pathophysiologic relevance to the cytotoxic event. Studies in interstitial nephritis have utilized both renal cell monolayers and tubular antigen–pulsed macrophages as targets for cytotoxic T cells [173, 174]. Other investigators have demonstrated cytotoxic T cells in biopsy [175] or nephrectomy specimens of rejecting human renal allografts [176, 177]. Modified ^{51}Cr release assays have also been utilized to test for natural killer cell activity [178–181] and antibody–dependent cell–mediated cytotoxicity by K cells [169, 182].

Neutrophils, monocytes, and macrophages accumulate at sites of tissue injury in large part because of their ability to display chemotaxis (directed locomotion) in response to chemical substances present in such areas. In general these chemoattractants interact with high affinity receptors on the surface of responding leukocytes [183–191]. The affinity of this chemoattractant–receptor interaction is not invariable and is affected by the chemoattractants themselves [192, 193] and other second messengers [194]. How the signal of an occupied receptor is translated into enhanced, directed cell locomotion is unclear. A number of biochemical events have been shown to accompany receptor binding, including alteration in membrane potential [195], mono- and divalent cation fluxes [196, 197], changes in cAMP and cGMP [198], and increased turnover of protein methyl esters [199]. Pharmacologic agents which inhibit methyltransferase reactions [200], phospholipase A_2 action [201], or the lipoxygenase pathway [188, 202] all serve to inhibit chemotaxis; addition of oxidized metabolites of arachidonic acid, in the latter example, will restore the chemotactic response. It is important to note that these chemoattractants, in addition to stimulating chemotaxis, appear to activate secretion of lysosomal enzymes and toxic O_2 metabolites by phagocytic cells [203, 204]. Thus, they may act in vivo as mediators stimulating potential nonspecific mechanisms of tissue damage.

There are two general techniques used to measure leukocyte chemotaxis. Less frequently used, because of economic and time constraints, is the microscopic visual observation of leukocytes migrating toward a chemotactic gradient source [205]. Ideally, such assays use time lapse cinematography to record cell migration. A modification of this assay, which is simpler and less expensive, is the recording of the orientation of cells in response to a chemotactic gradient [206]. Although these assays bear the advantage of direct observation of cell behavior, they are impractical. The more widely used assay, initially described by Boyden [207], utilizes chambers with two compartments separated by a filter [205, 208, 209]. One is a cellulose ester filter in which the chemoattractant gradient is formed. Cells migrate through this matrix both as function of their inherent locomotive properties and in response to the formed chemoattractant gradient. These assays can be utilized to test unknown fluids for chemoattractant activity, or cell populations for their ability to migrate in response to known chemoattractants. In either case, appropriate controls (to estimate the amount of unstimulated cell migration, and/or to document chemotaxis to defined agents when testing sera for activity), must be performed. Lastly, many clinical laboratories use an agarose assay to quantitate chemotaxis [210]. Cells in suspension are placed between wells containing control buffer or chemoattractant. Migration of cells through the agarose is measured in each direction. This assay is relatively simple, inexpensive, and often provides enough precision for clinical studies.

As with all in vitro assays, resulting chemotaxis cannot be easily extrapolated to in vivo conditions. For example, while the presence of a defined chemoattractant gradient across the kidney in experimental αTBM disease producing interstitial nephritis may be a relevant mechanism by which macrophages accumulate in this lesion [211], it does not rule out other explanations. Similarly, observed chemotactic abnormalities in patients with glomerulonephritis have unclear pathophysiologic relevance, as results are highly dependent on assay conditions [212].

In vivo correlates of T cell–mediated immunity

While highly refined in vitro systems are analytically useful in the evaluation of cell–mediated processes, the physiologic or pathophysiologic corroboration of these systems generally require analogous in vivo measurements. The in vivo reaction classically involving effector T cells is that of delayed–type hypersensitivity (DTH) to foreign proteins or contact sensitivity to chemical haptens. Landsteiner and Chase first demonstrated that contact sensitivity could be elicited in guinea pig recipients of immune cells upon challenge with the sensitizing agent [213]. Subsequent studies extended similar observations to the mouse [214, 215] and humans [216, 217], followed by investigations demonstrating that the effect was mediated by T lymphocytes [218, 219]. DTH reactions are mediated by antigen–specific T cells which induce a collection of neutrophils and monocytes at the site of intradermal challenge. Local tissue destruction is facilitated by a variety of soluble factors producing erythema and induration [220, 221]. The initial reaction in guinea pigs and humans usually occurs 24 hours after challenge, and classically begins with the appearance of neutrophils followed by a predominance of macrophages and occasional epithelial granulomas [216, 222–224]. In mice and rats the neutrophils tend to persist with the mononuclear presence [222]. The specific characteristics of the histology, however, are probably less important than the fact that the resulting induration is T

cell–mediated and antigen–specific. In humans, such effector T cells seem to be $T4^+$ [216], while in mice they can be either Lyt 1^+ $L3T4^+$, or Lyt 2^+ lymphocytes [218, 219, 225]. DTH reactions are particularly useful in experimental studies because immune cells can be characterized or admixed and transferred into naive recipients to evaluate T cell–effector function [225], or the mechanism of immune regulation [92, 101, 102, 114, 115].

Several specific considerations apply to the measurement of delayed–type hypersensitivity. The strength of the primary immunization and the length of time from exposure to antigen are important. These must be kinetically analyzed to optimize the test system. The strength of the challenging agent will also vary [223], but microgram quantities are often necessary in protein systems. The vehicle containing the antigen must be non-toxic. Although quantification of the DTH reaction can be performed by measuring the diameter of skin induration [222], increase in ear thickness [220], incorporation of ^{3}HTdR at a local site [226], and thigh or footpad swelling [225, 227, 228], it is the last of these, particularly in mice and rats, that has become especially popular. This may relate to the relative ease with which it can be learned [222]. Variability in measured results can be minimized by injecting all recipients at the same anatomic landmark without drawing blood. Quantitation of the reaction 24 to 48 hours after challenge can be performed with calipers [229], plethysmography [230, 231], or with a spring–loaded engineers micrometer [225, 232]. In our hands the latter instrument gives quite reproducible results. When footpad measurements are made, the investigator should be comfortably seated with the foot held under slight tension and perpendicular to the micrometer. It is critically important that the reader be blinded to the experimental groups.

The hypothesis that sensitized cells mediate an in vivo immune reaction can be formally tested in experimental animals using adoptive transfer protocols [213, 233]. These transfers must be made in syngeneic recipients to avoid acute rejection. Transfers into semi-syngeneic recipients are possible when the immunologic test is applied within a few days and when the possibility of acute rejection as an explanation for a negative result has been ruled out by demonstrating that immune cells from F_1 hybrids can transfer the immunologic effect into one of the parents. This latter strategy has been routinely employed in the study of MHC restrictions of DTH reactions [225]. Since most pathologic lesions take several days to develop, semi-syngeneic transfers are not really analytically useful. Appropriate experimental controls for adoptive transfer protocols would include the use of irrelevant immune cells, cells depleted of effector lymphocytes, and normal cells incubated with and washed of antigen to eliminate the possibility of antigen carryover. In addition to intravenous transfer, sensitized cells can be applied to a renal subcapsular location [234]. This approach allows an evaluation of the presence or absence of immune effector function in the transferred population, and circumvents issues of systemic migration or counter-regulatory events that might otherwise interfere with the transfer of effect [64, 225]. The acute results of such a transfer protocol can be defined kinetically by serial sacrifice for pathologic evaluation.

Bone marrow chimeras can also be constructed to explore the role of genetic factors in the pathogenesis of cell–mediated lesions [235]. Chimeric animals are prepared by lethally–irradiating recipients and reconstituting them within several hours with T cell–depleted bone marrow of cells of dissimilar origin. After 8 to 10 weeks the recipient is typically reconstituted with lymphoid cells of donor origin. This can be confirmed with antisera to allotypic markers of interest. Chimeras in which the recipient has, in addition to being lethally irradiated, also been previously thymectomized and/or reconstituted with donor thymus permits an evaluation of the thymic effect on subsequent cell–mediated events. The chimeric approach has been successfully used in evaluating the determinants of MHC restriction [236, 237], the nature of self–tolerance [237, 239], and the mechanism controlling disease expression in experimental renal lesions [240].

In situ evaluation of cell subpopulations in kidney lesions can be performed by immunofluorescence with antibodies to selected cell–surface determinants. Appropriate negative controls must be consistently used, and validation by eluting cells from tissue is valuable [241], but laborious. Ringed cell fluorescence can be confirmed with ethidium bromide staining of nuclei [242], and new anti-quenchers can be added to the glycerol coating of sections to prevent fluorescent fading [243, 244]. The optimal dilution of first and second antibodies can be standardized using cells from normal lymphoid organs. Properly done, these immunofluorescent analyses can provide useful, but static information regarding the constituents of local pathologic infiltrates [241, 245–247].

Experimental strategies

In working with cell–mediated T lymphocyte systems, either in vivo or in vitro, one is always challenged by the conditions which validate scientific reality. In many cases, the response to this challenge can only be an approximation of continuing refinement, and there are several considerations that currently should be kept in mind. A number of immune processes, as an example, depend on genetically–defined rules of cell interaction which regrettably limit many human studies, but are less of a problem with inbred experimental animals. The exact date of onset for many human renal diseases is often unknown, so that comparative differences among immunologic measurements requires a kinetic analysis for time–controlled interpretations. Cells displaced from their normal anatomic compartment and used in culture, or depleted of selected subpopulations, may also only be partially able to carry out their functions when placed in other environmental circumstances [248]. Purifying subpopulations of cells may furthermore reduce overall viability, and the purification process itself may unwittingly eliminate cells with high or very low affinity for particular antigens or determinants such that one's experimental results may only reflect a segment of the immune response that is of interest [249]. All of these issues can impact on the interpretation of collected data.

Identifying a relevant nephritogenic cell population requires the meeting of several specific criteria, not unlike those which would satisfy Koch's postulates. While regulatory and inducer T cells do not necessarily need to be present in a kidney infiltrate, it would be expected that directly mediating nephritogenic cells could be consistently isolated from local renal lesions, that their phenotype and specificity could be obtained, and that the adoptive transfer of such cells would reproduce the lesion in naive animals. Some cells within renal lesions, like

Table 1. Nephritogenic immune response profile: T lymphocyte interactions

Induction pathways	Immune regulation	Effector cell events
1. Immune response genes	1. Suppressor cell circuits	1. T cell–dependent antibody synthesis
2. Abrogation of tolerance	a. Auto–regulation	2. Effector T cells
3. Immune activation	b. Induced–regulation	a. Cytotoxicity
a. Accessory cell presentation		b. Delayed–type hypersensitivity
b. Helper cell–mediated differentiation of effector events		c. Lymphokines/histokines
		3. Macrophage events

neutrophils, macrophages, and natural killer cells, are typically just collected components of second–order inflammatory events. An immune cell, such as a T cell, has both specificity for an antigen and immunologic memory. It is these latter cells which will be the principle focus of the remaining discussion.

One of the largest problems in doing experiments with T cells is the proper assignment of specificity controls. Specificity controls can only be as good as the imagination of the investigator and the biochemical precision with which the relevant antigen has been defined. When the relevant determinants are from renal tissue, the best specificity controls are renal tissue minus the determinant, relevant determinants with minor modification, or perhaps, similar tissue from another parenchymal organ from the same host. At a minimum, to be fair, they should be moieties with which a cell population might have had some potential exposure to but, in fact, have had no experience with. Another troublesome area is the inference of T cell function from cell–surface phenotype. While there are a few exceptions to the rule, T cell phenotype generally only indicates that MHC context within which T cells recognize antigen [17]. Nothing really meaningful can be said about function by knowing phenotype, just as nothing about function can be construed from simple measurements of T cell proliferation [144]. There are many different immunologic techniques which can be used to probe the role of T cell immunity in renal disease. The general area of cellular immunity can be divided into three arbitrary domains (See Table 1).

Induction pathways

Investigations in this area examine the origins of the nephritogenic immune response. Whether the principle, local effector mechanism is humoral or cell–mediated, both have a beginning with antigen–recognition, overcoming tolerance, and immune activation. For many immune responses the induction pathway appears to be the primary level of immune response gene effect reflecting disease susceptibility. There are several genetic regions which are frequently involved in T cell recognition of antigen. In the mouse they are the H-2, Igh-1, and IgT-C regions [3, 19, 20]. It is equally likely, however, that a nephritogenic immune response can map to additional genetic loci for which no recombinant event has yet provided a measure of distinction, and as such, are often referred to as non-MHC-linked [250–252]. One can test for many of these possibilities by using genetic recombinant or congenic mice and rats. Once the lesion has been reproducibly established in an inbred rodent species, then other strains can be selected which differ in the MHC region (or subregion), at Igh-1, IgT-C, or in the background of the susceptible strain. The ability to detect differences among selected recombinants, of course, assumes that allelic variations exist within the species; that is, some strains will get disease while others will not, otherwise no such assessment can be made. If the nephritogenic immune response is directed towards a renal parenchymal antigen, one can also potentially map for its genetic location by using phenotypic markers suggested by the characteristics of the strains which do or do not express the renal antigen of interest (such as albinism, pink–eye, or a liver enzyme of established location) [253]. If the F_1 hybrid expresses the antigen, these mapping studies are statistically more powerful when done as F_1 backcrosses to non-susceptible parents rather than as F_2 segregation studies. In human experiments, genetic linkage is often established by assigning association probabilities, relative risks, and etiological fractions using statistical models [254].

Nude athymic rodents can also be used to determine if disease activation is a T cell–dependent event, assuming that the heterozygote littermates are disease–susceptible [255]. If athymic rodents of a susceptible strain are not available, then thymectomized, lethally irradiated, T cell–depleted bone marrow reconstituted rodents can be used several months after preparation (B-cell mice or rats) [256]. Radiation–induced bone marrow chimeras (susceptible $\times$ non-susceptible) $F_1 \rightarrow$ non-susceptible recipients, or vice versa) can also be used to analyze the role of host–environment or thymus–origin on the ability of T cells to respond to selected antigens [257–259]. The actual measurement of T cell effect depends, to a certain degree, on the nature of the response in the non-susceptible host; that is, if no response to antigen is made, then a simple proliferation assay can be used to measure antigen–recognition. If only a distinctive qualitative difference in susceptibility exists in the non-susceptible strain, then a functional assay (T cell help for antibody production, or T cell cytotoxicity, or delayed type hypersensitivity; vide infra) will be a necessary requirement.

When the nephritogenic immune response is directed towards a renal alloantigen, it is likely that the susceptible host has had to overcome normal tolerogenic mechanisms preventing spontaneous self–aggression [260]. Generally, for example, a susceptible host will not ordinarily make a meaningful immune response to autologous antigens, unless the dose is unusually large or the adjuvant unusually strong, while the host may easily respond to the same nephritogenic determinant presented in a heterologous format. To study the vagaries of tolerance, and to maximize the likelihood of identifying an operative-tolerogenic mechanism, the primary immunization should be sufficient, but not so overwhelmingly strong as to make any form of tolerance functionally inoperative [261]. While a variety of tolerogenic mechanisms have been proposed or characterized, many of the functionally important ones seem to involve the T cell repertoire [262]. They include, in the non-susceptible

host, the failure of antigen to associate with MHC class II alleles on antigen-presenting cells, an absence of T cells which can respond to antigen, and the presence of active and specific T cell suppression [262]. These issues can best be addressed by using T cell lines [263], antigen-pulsed presenting cells [264], F_1 hybrids or chimeras [258], or in the case of suppressor cell-mediated clonal silence, by using low-dose irradiation [265], antigen-suicide [266], or cyclophosphamide treatment to abrogate functional deletions [92, 101, 260]. Add-back adoptive transfer experiments with selected subpopulations of cells can then be employed to characterize and confirm the relevant cells mediating the tolerogenic effect.

The induction process finally culminates in the development of an immune effector repertoire. For most immune responses to complex antigens, there are a series of cell interactions involving T helper/inducer cells mediating the differentiation of B cells, cytotoxic T cells, and delayed-type hypersensitivity responses (infra vide); that is, T helper cells would not necessarily be the direct mediator of a nephritogenic effect. The interactions leading to such an effect can, however, be analyzed using in vitro cell culture-induction systems [64, 101, 267–269]. Requirements for a differentiating effect may vary, but generally include a source of T helper cells (often as a stable line or clone), T and/or B cell growth factors, and a source of naive effector precursor and antigen-presenting cells. The resulting humoral or cell-mediated effector event can then be passively or adoptively transferred to measure nephritogenic potential [64]. Such assay systems can also be used to analyze the role of lymphokines, soluble helper factors, genetic restrictions, and mechanisms of phenotypic selection and acquisition of receptor affinity. In vitro induction assays are particularly useful paradigms for evaluating and characterizing the biochemical mediators of immunologic differentiation. Helper cell factors from cell lines or T cell hybridomas can be run over immunoaffinity columns with specificities for the primary ligand, MHC subregion determinants, and idiotypes, and the different fractions collected can then be tested for inductive properties [90, 96].

Immune regulation

Studies in the area of immune regulation can provide evidence for the mechanisms which control or limit nephritogenic immune responses as well as provide opportunities to modulate the natural history of ongoing renal disease [1]. Most regulatory systems intrinsically utilize subpopulations of T cells which can induce or effect suppression as well as provide contrasuppression, or second level inhibition of suppression [91, 92]. These networks depend on complementary interactions between antigen, idiotype, and antigen-presenting cells, are restricted by gene products in the major histocompatibility complex, and have been thoroughly reviewed elsewhere [1, 91, 92, 115]. A regulatory process can theoretically influence the development of immune-mediated renal disease in two general ways. First, a pluri-potential component cell or network in a normal regulatory pathway can cease to operate or fail to develop such that any number of autoreactive immune responses would spontaneously arise to produce mediators of renal injury. Second, an expected regulatory event in response to a nephritogenic antigen could be delayed or fail to appear. Under these latter conditions, the immune response cannot be terminated, and what would be otherwise a self-limited event now becomes a progressive form of renal injury. Regulatory networks can also be divided into those which are investigator or externally-induced and those which naturally arise as self-limiting events.

One experimental strategy that can be used to identify the presence of a natural or ontologic suppressor cell effect is to co-treat a susceptible recipient with low-dose irradiation, or cyclophosphamide and immunize with: 1) a subnephritogenic dose of antigen; 2) a renal antigen which normally does not produce disease in an autologous format [101]; or 3) a full-strength antigen that produces worse disease when kinetically compared to similar recipients who are not co-treated [102]. Using one of these constructed paradigms, relevant naive T cell subpopulations can then be added back to abrogate the heightened effect. Similar kinds of experiments could be performed with nude, or B cell mice or rats reconstituted with a nephritogenic effector mechanism [256, 270]. In mice with spontaneous interstitial nephritis, there is an attrition of tubular antigen-specific suppressor T cells which can be restored by adoptive transfer of lymphocytes from normal congenic strains [240]. In viral glomerulonephritis in weanling mice, there is a failure in the development of suppressor T cells which can be corrected by adoptive transfer protocols using adult infected mice [271]. F_1 hybrids from susceptible and non-susceptible mice have also been used to illustrate the presence of a regulatory network (T cell and anti-idiotypic) inoperative in the autoimmune susceptible strain [105]. Suppressor or regulatory mechanisms can also be analyzed using functional assays measuring effector events like antibody formation [106, 107] or effector T cell-mediated delayed-type hypersensitivity after local passive transfer [64]. Such assays have been used to evaluate the role of ligand-specific suppressor cells in mercuric chloride glomerulonephritis [106, 107], IgA-nephropathy [108], lupus mice [103, 109], Heymann nephritis [104], and experimental interstitial nephritis [114].

In addition to the study of naturally-occurring suppressor cells which appear as a self-limiting mechanism during the course of renal disease, it is also feasible to employ exogenous, or investigator-induced suppressor networks which are constructed as therapeutic probes to measure externally applied regulatory effects. Generally, these experimental strategies are initiated by inducing antigen or idiotype-specific suppressor cells in a naive syngeneic animal, and then adoptively transferring the donor cells either at the time of recipient immunization or, more importantly, after the disease process has been initiated. These cell-mediated suppressor networks typically involve several sets of sequentially activated suppressor T cells which have complementary specificities. They often operate under genetically-defined rules, have interactions with subpopulations of antigen-presenting cells, and they usually secrete soluble suppressor factors which mediate complex interactive functions [91, 92]. To be of interest, such suppressor networks must have an inhibitory effect on the renal disease in question. It will not always be possible, however, to use disease as an end point in all experiments attempting to characterize fine-specificity or genetic restriction. This is most easily accomplished in short-term assays measuring relevant nephritogenic effector events, like the inhibition of production of antibodies or effector T cells [106, 107, 114]. Such assays can be performed by admixing before plaquing [65, 106], or by quantitating the

effect of suppressor T cells, or their soluble suppressor factors, on the development of antigen–specific delayed–type hypersensitivity [64], or cytotoxicity [273, 274]. Characterization of specificity, complementary idiotype, phenotype, and genetic restriction are easily analyzed with such paradigms [92]. Suppressor cells can be induced with antigens in incomplete Freund's adjuvant [112, 113, 275], by hyperimmunization with T lymphoblasts [110, 111], or by using intravenously injected ligand–coated lymphocytes [114]. With the latter method, it has also been possible to directly induce auto-anti-idiotypic suppressor cells in mice with active renal disease. Suppressor cells established through such strategies may be purified by ligand–panning (as suppressor cells seem to be able to directly bind ligand), started in long–term culture as suppressor T cell lines [65, 69], or fused with T cell lymphomas to make factor–secreting hybrids [75, 76].

Effector cell events

The identification of relevant T cell–mediated or T cell–dependent effector mechanisms is critical to the dissection of many local nephritogenic events. If such processes can be distilled to their functional essence they can also become useful probes into the inductive and regulatory processes discussed above. In nearly all experimental strategies putative nephritogenic T cells will have to be able to either adoptively transfer the inflammatory reaction into naive recipients or, in the case of T-B cell interactions, induce the production of nephritogenic antibodies in culture which can then passively transfer the lesion.

The adoptive transfer of immune effector T cells, while not always necessary [275, 276], may be enhanced by prior in vitro culture with antigen [63, 64, 277] and, in the case of T cell lines, may require subsequent administration of exogenous IL-2, or sub-lethal irradiation of the recipient to produce lesions. Such manuevers to transfer disease in naive recipients are not an invalid test of efficacy, as the naive host is not a conditioned, nor necessarily a conducive environment for immunologic effector cell expression. This may be particularly true when the antigen is parenchymal self. Exogenous planted glomerular antigen, on the other hand, seems to be easily recognized by circulating immune lymphocytes [278, 279]. In the case of the kidney, it is technically possible to place immune reactive cells under the capsule to assess direct nephritogenic effect [64, 225, 243]. In some cases this may be facilitated by using soluble antigen in the transfer vehicle. If effector T cells recognize their antigenic epitopes in the context of MHC determinants, then the expression of such determinants may also modulate the efficacy and rapidity with which transferred cells localize within the renal parenchyma. Non-specific activators of MHC class antigens on somatic renal cells, like interferon, may be useful in priming the naive recipients [280].

With the variety of antibodies to cell–surface determinants that are presently available, it is now also feasible to phenotypically characterize the inflammatory cells comprising a renal lesion of interest [241, 245–247]. While such studies form an initial data base of information, they, of themselves, do not constitute a definitive statement of mechanism. Implying function from phenotype is inherently uncertain and many times incorrect [17]. It is possible, however, to isolate infiltrating cells, positively or negatively select subpopulations of interest, and apply them to assays for functional effect [64].

There are several functional assays which can be employed to obtain useful quantitative results. In evaluating T-B cell interactions, there are plaquing assays for measuring B cell synthesis of antibody to antigen or idiotype, or there are radioimmunoassays which can directly measure synthesized antibody of interest [150–161]. Effector T cell function can be analyzed by in vitro cytotoxicity assays [173, 174, 281] if enough effector cells are available, and if the relevant target antigen can be covalently linked or expressed by a chromium–labelled cell that has an acceptably low level of spontaneous release. In some cases it may be appropriate to use labelled renal parenchymal cells as a target [173]. Isolated immune T cell populations can also be assessed for effector function in assays measuring delayed–type hypersensitivity. Delayed–type hypersensitivity reactions can best be utilized in adoptive transfer strategies where immune cells are injected intravenously or locally into the footpad [64, 225]. This latter method is particularly useful when there are limited numbers of cells. The precision of the measurement, and the clonal nature of these interactions are, perhaps, best evaluated with cell lines where stability, manipulability, and predictability are achieved with greater reliability. Human studies are limited to measurements of T cell function using assays of antibody synthesis or cytotoxicity [61, 155–158], and the current ability to grow lymphocytes out of renal biopsies holds promise for establishing useful human cell cultures for immunologic analysis [175]. Lymphocyte culture systems for both animals and humans also provide a ready source of lymphokines for molecular and immunologic study [83, 90, 121]. Cell lines or initial cultures can be fused with a variety of lymphomas, and once stabilized, can often produce substantial quantities of such factors [75, 76].

Finally, macrophages are a common cell type found in both glomerular and interstitial nephritides. The presence of such cells can be verified by antibodies to cell–surface determinants [246] or by special stains [241] and, in histocompatible murine systems, the origin of localizing macrophages can be tracked with distinctive bone marrow cells from Chediak–Higashi mice [282]. The generation and presence of renal–derived chemoattractants can also be kinetically analyzed using arterial and renal venous serum samples obtained across nephritic kidneys [211]. The role of macrophages in hypercellular lesions are verifiable by pretreatment protocols using anti-macrophage serum [283, 284], with the effect of antibody Fc-piece on macrophage elicitation can be analyzed with comparisons between intact immunoglobulin and $F(ab')_2$ fractions [285].

Summary

This overview has examined some of the current experimental options available for the study of cellular immunity in the immunopathogenesis of renal disease. T cell immunity, where it has been examined, seems to have a particularly pivotal role in orchestrating and regulating functional patterns of renal injury. The use of the research methods presented here for the study of cell–mediated interactional events in kidney disease, however, has lagged behind similar efforts in other organ systems. We hope, therefore, this report will serve to stimulate and strengthen further interest in the cell biology of the nephritogenic immune response.

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