ANTIIDIOTYPIC IMMUNITY IN INTERSTITIAL NEPHRITIS

II. Rats Developing Anti–Tubular Basement Membrane Disease Fail to Make an Antiidiotypic Regulatory Response: the Modulatory Role of an RT7.1$^+$, OX8$^-$ Suppressor T Cell Mechanism

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Brown Norway rats immunized with renal tubular antigens in adjuvant develop a severe interstitial nephritis (1–3). The expression of disease is characterized by the appearance of anti–tubular basement membrane antibodies (αTBM-Ab)[1] followed by an intense mononuclear cell infiltrate. The cellular lesion is composed of several subsets of T lymphocytes, macrophages, and Ig$^+$ cells (4). Natural killer cells have also been observed in the murine form of disease (5). The relevant tubular antigen for disease is a glycoprotein expressed on cortical tubular basement membranes (Neilson and Kefalides, unpublished observations) by genes closely linked with albinism on the first linkage group (1). The most important immunologic response to this tubular antigen is cell mediated, and is largely determined by an RT1-linked gene. There are strain differences in the amount of αTBM-Ab produced, but the genes involved are not RT1 linked and the antibody titer seems to have little or no effect on disease susceptibility (1).

If rats are pretreated with tubular antigen-reactive T lymphoblasts in adjuvant and then immunized to produce disease, they do not develop interstitial lesions (6). Protected animals show a decreased T cell proliferative response to tubular antigens, produce αTBM-Ab with a reduced binding affinity for tubular antigen, and develop antiidiotypic antibodies that bind to idiotypes within the antigen-binding variable region of monoclonal αTBM-Ab. These results suggest that antiidiotypic immunity might have a modulating influence in this model both at

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[1] *Abbreviations used in this paper:* αTBM, anti–tubular basement membrane; αTBM-Ab, αTBM antibody; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; Con A, concanavalin A; DTH, delayed-type hypersensitivity; EDCI, 1-ethyl 3-(diethylaminopropyl)carbodiimide; HGG, horse gamma globulin; [^{3}H]TdR, tritiated thymidine; NWA, nylon wool adherent cells; NWT, nylon wool T cells; PBS, phosphate-buffered saline; PBS-5, 5% fetal calf serum in PBS (pH 7.2); PPD; purified protein derivative; PVC, polyvinyl chloride; RE-αId, antiidiotypic antibodies against RE-Id; RE-Id, polymorphic TBM-Ab eluted from nephritic kidneys; RTA, renal tubular basement membrane antigen; SLA, soluble liver antigen; SRTA, soluble renal tubular antigen; TB-αId, antiidiotypic antibodies against tubular antigen-reactive T lymphoblasts.

the level of antigen recognition and in the development of antigen-receptor heterogeneity.

In the present studies we have further observed that rats normally immunized to produce disease do not make a humoral or cell-mediated antiidiotypic immune response to αTBM-Ab. Antiidiotypic immunity can be generated, however, if the rats are pretreated with low-dose cyclophosphamide before immunization. Because nephritic rats also develop nonspecific suppressor cells early in the αTBM immune response, we evaluated the effects of this suppressor system on the generation of antiidiotypic immunity. We now report that a cyclophosphamide-sensitive, $RT7.1^+$, $OX8^-$ suppressor T cell inhibits the formation of such an antiidiotypic effect. Furthermore, tubular antigen-reactive cells unencumbered by this suppression can induce a protective antiidiotypic immune response in naive animals. These findings suggest that suppressor T cells in the afferent phase of this nephritogenic immune response can modulate subsequent immunoregulatory events.

Materials and Methods

Animals. Brown Norway rats obtained from the Charles River Breeding Laboratories, Wilmington, MA were maintained by the Department of Laboratory and Animal Medicine, University of Pennsylvania School of Medicine.

Preparation of Renal Tubular Antigen. Rabbit renal tubular basement membrane antigens (RTA) and rabbit liver segment membrane antigens were isolated by a differential seiving technique (7). Highly enriched basement membrane fragments were sonicated, lyophilized, and stored at −70°C. Soluble renal tubular antigens (SRTA) and soluble liver antigen (SLA) were made from these lyophilized membranes using collagenase digestion (1, 8).

Induction of Disease. Groups of rats were immunized with 2 mg of RTA in complete Freund's adjuvant (CFA) by footpad and/or subcutaneous injections (1, 6). Control animals received CFA alone. Some rats were pretreated with 50 mg/kg of cyclophosphamide 48–72 h before their immunization (9).

Assessment of In Vitro Lymphocyte Proliferation. Cells from draining lymph nodes were placed in suspension in RPMI 1640 supplemented with antibiotics, L-glutamine (2 mM), *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (10 mM), 2-mercaptoethanol (5×10^{-2} mM), and 5% heat-inactivated fetal calf serum (1). 2×10^5 cells in 0.2 ml of complete media were co-cultured in triplicate microtiter wells at 37°C in 5% CO_2 in the presence or absence of concanavalin A (Con A) (2.5–10 μg/ml), SRTA (5 μg/ml), SLA (5 μg/ml), or purified protein derivative (PPD) (10 μg/ml). Cultures were labeled with [^{3}H]thymidine ([^{3}H]TdR) during the last 4 h before harvesting for scintillation counting. The Con A cultures were harvested on day 3 and all other cultures were harvested on day 4. The ratio of mean cpm from stimulated cultures per mean cpm from control cultures was used to calculate a stimulation index ± SEM.

Assessment of In Vitro Suppressor Cell Activity. The mitogen-stimulated suppressor cell assay has been previously described in detail (7). Briefly, 1.25×10^5 irradiated (3,000 rad) immune or control lymphocytes were co-cultured (1:1 ratio) with third-party lymphocytes from normal animals used as responder cells. Con A was added to those wells designated for stimulation and media was added to control wells. Cultures grown in flat-bottom microtiter plates for 72 h at 37°C were assayed for blastogenesis by adding [^{3}H]TdR. The results were expressed as a stimulation index or as a percent suppression using a previously described formula (7).

Preparation of T Cells and Their Subpopulations. Cell suspensions were depleted of T lymphocytes with a monoclonal IgM and anti-RT7.1 antibody (from BC84.5 hybridoma [10–12] provided by Dr. John Ely and Dr. Frank Fitch, University of Chicago, Chicago, IL) and a mixture of rabbit and guinea pig complement. By microcytotoxicity assay, this

antibody kills 95% of thymocytes, 58–62% of lymph node cells, and 33–35% spleen cells. Nylon wool T (NWT) and nylon wool adherent (NWA) cells were obtained from packed nylon wool columns (3–4 × 10^8 cells added to a 35-ml column) according to the methods of Julius et al. (13). Monoclonal antibody OX8 (cytotoxic/suppressor T cell subset) was purchased from Accurate Chemical & Scientific Corp., Westbury, NY (14). The OX8 monoclonal antibody was used to enrich for suppressor T cells by an indirect panning technique (15, 16). Briefly, 2.5 ml of affinity-purified rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA) in a concentration of 1 mg/ml in phosphate-buffered saline (PBS) was incubated on 60 × 15-mm polystyrene dishes overnight at 4°C. After removal of the Ig solution, the plates were washed and blocked with 5% fetal calf serum in PBS (PBS-5) for 30 min. The fetal calf serum was then washed out before the addition of the T cells. 25 × 10^6 NWT were preincubated in 0.2 ml of a 1:40 dilution of OX8 antibody (ascites fluid) for 30 min at 4°C. After extensively washing the cells in PBS, they were transferred onto the anti-IgG–coated dishes for 60 min at 22°C. Midway through the incubation the dishes were gently rocked. Nonadherent cells were removed from the plates with three cycles of swirling and washing with PBS-5. After the nonadherent cells were removed, 3 ml of chilled PBS-5 was added to the dishes, which were then placed at 4°C for 30 min. The adherent cells were then resuspended by vigorous pipetting and further washed with chilled PBS-5. The presence of Ig^+ cells in the separation mixture was determined by direct immunofluorescence using RITC-anti-rat Ig (gift of Dr. Susan Webb, Wistar Institute, Philadelphia) and the percentage of $OX8^+$ cells was determined by indirect fluorescence using FITC-anti-mouse IgG (Cappell Laboratories, West Chester, PA).

Preparation of Idiotypes. Monoclonal anti–tubular basement antibodies BN52/19 (IgG1) and BN48/12 (IgG2a) were derived from rat-mouse fusions as previously described (6). Purified preparations of these antibodies were made from cell cultures grown in serum-free media for 36 h. The media was cut with 50% ammonium sulfate and the pellet was resuspended in distilled water and extensively dialyzed against PBS. These preparations contained >90% IgG by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polymorphic idiotypes (RE-Id) were prepared by acid eluting αTBM-Ab from nephritic kidneys using glycine-HCl (pH 2.6) (17). The pH of the eluate was neutralized to pH 7.3 and further cut with 50% ammonium sulfate. Affinity-purified eluate was prepared by passing the sulfate-precipitated antibodies over an affinity column made with anti-rat IgG coupled to activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) (18). These polymorphic αTBM-Ab were then eluted from the affinity column with glycine-HCl (pH 2.6).

Preparation of Antiidiotypic Antibodies. Two antiidiotypic antibodies were independently prepared. TB-αId antibodies were derived from the serum of BN rats immunized with tubular antigen-reactive T lymphoblasts as previously described (6). Low-titered αTBM-Ab were removed from this preparation by extensive adsorption with tubular basement membranes. RE-αId antibodies were prepared by immunizing a rabbit with polymorphic αTBM-Ab (RE-Id) that had been eluted from nephritic kidneys. This hyperimmune serum containing RE-αId antibodies was extensively absorbed against BN gammaglobulin coupled to activated Sepharose 4B (18). The specificity of their binding was determined by competitive inhibition radioimmunoassays using SRTA, SLA, relevant idiotype, and control antibodies. To determine the specificity of the TB-αId antisera, polyvinylchloride (PVC) plates coated overnight with 25 μl of the antisera at a dilution equal to 50% binding reactivity to RE-Id. After blocking with 4% bovine serum albumin (BSA), several potential inhibitors were coincubated at several dilutions for 2 h with ^{125}I-RE-Id. To determine the specificity of the RE-αId antisera, RE-Id was coated on PVC plates overnight at a concentration of 25 μg/ml. After blocking, RE-αId at 50% binding reactivity was then coincubated with several potential inhibitors for 3 h. After three washes, this assay was developed with ^{125}I-affinity-purified anti-rabbit IgG. Individual wells from both assays were cut from the PVC plates for counting and the results were expressed as a percent of maximum binding.

Screening Assay for Antiidiotypic Antibodies. This solid-phase screening assay for antiidi-

otypic antibodies has been previously described (6). Briefly, optimum concentrations of cold idiotype (either BN52/19 or RE-Id) in 0.02 M PO_4 buffer were incubated overnight at 4°C in PVC plates. The plates were washed and blocked for 1 h with 4% BSA. Serial dilutions of pooled test antisera (25 μl) were then incubated at 22°C for 3 h. After further washing, ^{125}I-idiotype was added and the incubation continued for an additional 2 h. Individual wells were then counted and the results expressed as a percent of maximum binding.

Preparation of Idiotype-coupled Cells. Idiotypic antibodies were coupled to normal, syngeneic spleen cells using a carbodiimide procedure as previously described (19). Briefly, BN spleen cells were treated with Tris-buffered 0.83% ammonium chloride (pH 7.2) to lyse the erythrocytes. 4×10^8 cells were incubated with 1 mg of BN52/19 and 25 mg of 1-ethyl 3-(3-diethylaminopropyl)carbodiimide (EDCI) in 1 ml of 0.9% saline. The mixture was gently rotated at 4°C for 90 min and then gently washed three times with normal saline. Using these conditions, ~19.4 μg of BN52/19 (trace labeled with ^{125}I) was bound to 5×10^7 viable cells. In some experiments, horse gamma globulin (HGG) was also linked to normal spleen cells. Cell viability at the end of this coupling procedure averaged ~80% by trypan blue exclusion.

Induction and Elicitation of a Delayed-type Hypersensitivity (DTH) Response to Idiotype and Tubular Antigen. DTH responses to BN52/19 or HGG were induced by subcutaneously immunizing naive rats with 2.5×10^7 BN52/19- or HGG-coupled spleen cells in each flank (19). DTH responses to RTA were induced by subcutaneously immunizing rats with RTA in CFA (20). Five to six days after such immunizations, groups of rats were challenged in one footpad with 50 μg of soluble antigen (either BN52/19, RE-Id, BN48/12, control Ig, HGG, SRTA, and SLA, depending on the experiment) in 50 μl of PBS. Swelling as an index of DTH was measured 24 h later using a spring-loaded engineer's micrometer (Schlesingers for Tools Ltd., Brooklyn, NY). The magnitude of swelling was expressed as the increment between the challenged footpad compared with an unchallenged (or in most cases PBS challenged) footpad in inches $\times 10^{-3}$. All measurements were cage-blind. In selected experiments, histologic sections of swollen footpads revealed mononuclear cell infiltrates typical of DTH reactions.

Adoptive Transfer Experiments. Draining lymph node cells, spleen cells, or pooled mixtures were harvested 5–22 d after various immunization protocols. Single-cell suspensions were washed, counted, and intravenously injected ($1.2–10 \times 10^7$ cells/rat) using PBS as the vehicle. In some experiments, these cells were separated into various subpopulations (NWT, NWA, NWT/OX8$^-$, NWT/OX8$^+$, and T cell depleted with αRT7.1 plus C′).

Assessment of Renal Disease. Kidney tissue was prepared for light microscopy by standard methods for this laboratory (1). The degree of interstitial involvement was qualitatively graded from 0 to 4 on coded sections and expressed as a mean ± SEM for each group.

Statistical Analysis. Differences between experimental groups were determined by Student's *t* test.

Results

Assessment of the Antiidiotypic Immune Response in Nephritic Rats. In an introductory series of experiments, we wished to determine if rats normally immunized to produce disease would also demonstrate some measure of antiidiotypic immunity towards a relevant spectrum of tubular antigen-reactive immune products. Two screening assays were developed to detect either a humoral or cell-mediated antiidiotypic immune response. The most convenient and isolable source of idiotype in this model is αTBM-Ab. We reasoned that αTBM-Ab eluted from nephritic kidneys (RE-Id) might constitute an important source of polyclonal and polymorphic idiotype because the eluate reflects a relevant spectrum of tubular antigen-binding reactivity (21). In preparation for the serum-screening studies, two antiidiotypic antisera were independently prepared for use as positive

controls. TB-αId antibodies were made by hyperimmunizing BN rats with syngeneic tubular antigen-reactive T lymphoblasts (6). RE-αId antibodies were prepared in a rabbit hyperimmunized with RE-Id. Neither of the antiidiotypic antibodies bound normal IgG or SRTA by radioimmunoassay, nor did they stain tubular basement membranes by indirect immunofluorescence (data not shown). In Fig. 1*A*, we observed in specificity studies that RE-αId antisera was specifically inhibited by RE-Id antibodies, and by SRTA. Quite similar findings were also observed in the specificity studies for TB-αId antibodies (Fig. 1*B*). These findings by competitive inhibition criteria suggest that both antiidiotypic antisera were primarily directed towards idiotypes in or near the antigen-binding region. Furthermore, in Fig. 1*C*, low dilutions of TB-αId antisera significantly inhibited the binding of RE-αId to RE-Id when compared with control sera from nephritic rats that had been adsorbed to remove αTBM-Ab. This latter inhibition study suggests that the tubular antigen-reactive T and B cell repertoires possibly share some serologically detectable determinants that may be idiotypes. We have previously reported (6), in addition to the present studies, that TB-αId antisera binds monoclonal αTBM-Ab BN52/19 in or near its antigen-binding region. By competitive inhibition radioimmunoassay, BN52/19 represents ~13% and BN48/12 3–4% of the maximum inhibitable binding reactivity expressed by RE-Id for RE-αId (data not shown).

In direct-binding screening experiments, the sera from nephritic rats were examined for the presence of antiidiotypic antibodies against αTBM-Ab. In Fig. 2, nephritic sera were analyzed over a 100-d interval. No binding to BN52/19 (Fig. 2*A*) or to RE-Id (Fig. 2*B*) was observed with these pooled interval sera compared with the positive controls. Interestingly enough, however, antiidiotypic binding could be demonstrated on day 28 if rats were pretreated with low-dose cyclophosphamide before their immunization with RTA/CFA. Although the data is presented as pooled sera, individual sera from three cyclophosphamide-pretreated rats also exhibited similar binding reactivity. If the pooled antisera was absorbed against an anti-rat IgG column, its binding to BN52/19 was reduced to control values, suggesting that the positive binding effect was not due to excess antigen in that particular sera (data not shown). Furthermore, if

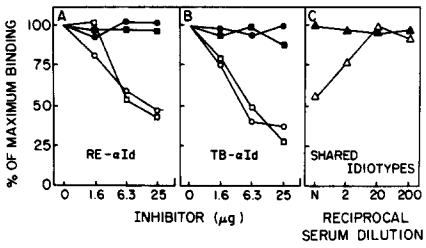

FIGURE 1. Specificity and cross-reactions of antiidiotypic antisera. (*A*) The specificity of RE-αId antibodies was demonstrated by competitive inhibition studies using SRTA (□), SLA (■), RE-Id (○), and control IgG (●). The findings demonstrate that RE-αId antibodies are inhibited from binding to RE-Id in the antigen-binding region; (*B*) TB-αId antisera was also specifically inhibited from binding to RE-Id, in a similar fashion; (*C*) TB-αId antisera (△) in low dilutions inhibited the binding of RE-αId to RE-Id when compared with control sera from nephritic rats depleted of αTBM-Ab (▲), suggesting partial cross-reactivity.

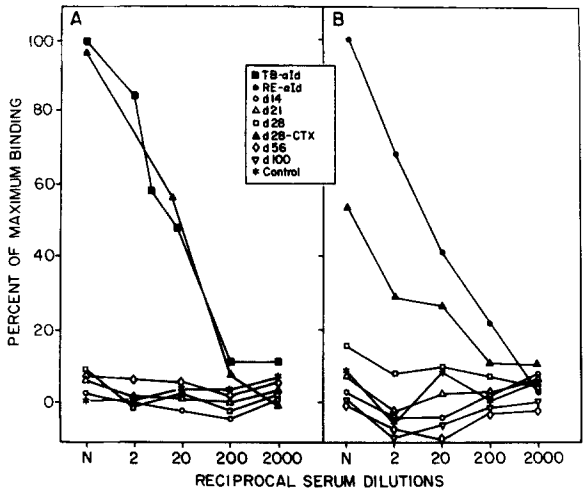

FIGURE 2. Direct-binding screening assay for antiidiotypic reactivity. (*A*) Pooled interval sera from five to seven rats per group with interstitial nephritis were tested for binding reactivity to monoclonal αTBM-Ab BN52/19. None of the interval sera showed any reactivity when compared with positive control sera (TB-αId). However, binding was demonstrated with sera taken from nephritic rats pretreated with cyclophosphamide; (*B*) Interval sera were also tested for binding to RE-Id. Antiidiotypic reactivity was only observed in the positive control (RE-αId) and in sera obtained from nephritic rats pretreated with cyclophosphamide.

TABLE I

DTH Response to Polymorphic αTBM-Ab Idiotypes and to Tubular Antigen

Immunization	DTH Response*				Histology§
	RE-Id	Control IgG	SRTA	SLA	
RTA/CFA	3.0 ± 0.3	2.8 ± 0.3	24.3 ± 2.4‖	3.1 ± 0.2	2.0 ± 0.3
CTX-RTA/CFA‡	15.5 ± 0.8‖	3.0 ± 0.3	24.7 ± 2.2‖	3.4 ± 0.3	3.2 ± 0.4¶
CFA	2.3 ± 0.3	2.8 ± 0.2	3.8 ± 0.4	3.3 ± 0.5	0.0 ± 0.0
—	3.0 ± 0.2	3.5 ± 0.4	3.8 ± 0.5	3.2 ± 0.2	—

* Data points from 6 to 11 rats per measurement were calculated as a mean footpad increment in inches times 10^{-3} ± SEM, 22 d after immunization, 24 h after challenge.

‡ 50 mg/kg of cyclophosphamide (CTX) was administered 3 d before immunization.

§ Severity of the interstitial lesions was qualitatively graded from 0 to 4 and expressed as a mean ± SEM.

‖ $P < 0.001$ compared with unimmunized controls.

¶ $P < 0.04$ compared with RTA/CFA group.

the plate-coated BN52/19 was replaced by control IgG, binding was also reduced by 90%. This latter finding suggests that the positive result observed with antisera from cyclophosphamide-pretreated rats cannot be explained by the presence of rheumatoid factors.

We also examined the possibility that an antiidiotypic immune response to RE-Id might be more easily demonstrated in nephritic rats as a cell-mediated reaction. In Table I, two groups of rats were subcutaneously immunized to produce disease. At 22 d, they were footpad challenged with tubular antigen (SRTA), polymorphic idiotype (RE-Id), or with specificity controls. The findings in this

TABLE II
Transfer of DTH to Polymorphic αTBM-Ab Idiotypes

Donor cells*	*n*	Treatment	DTH response‡	
			RE-Id	Control IgG
CTX-RTA/CFA§	6	—	17.1 ± 0.5‖	3.8 ± 0.3
CTX-RTA/CFA	4	C′	16.3 ± 0.6‖	4.3 ± 0.3
CTX-RTA/CFA	4	αRT7.1 + C′	6.3 ± 0.5	4.0 ± 0.4
CTX Control	5	—	4.2 ± 0.4	5.0 ± 0.5
Control	5	—	3.8 ± 0.6	3.8 ± 0.6
—	5	—	3.3 ± 0.4	3.6 ± 0.4

* Donor lymph node cells harvested from rats 21 d after immunization were transferred into naive recipients.
‡ Recipients were challenged 4–6 h after transfer and the mean footpad increment in inches times 10^{-3} ± SEM was determined 24 h later.
§ 50 mg/kg of cyclophosphamide (CTX) was administered 3 d before the immunization of donor animals.
‖ $P < 0.001$ compared with unimmunized controls.

table suggest that rats immunized with RTA/CFA make a specific DTH reaction to tubular antigen, but not to RE-Id. Similar findings were observed at days 32 and 43 (data not shown). If, however, immunized animals were pretreated with low-dose cyclophosphamide, such a DTH reaction to polymorphic idiotype could be observed. As shown in Table II, this DTH effect to RE-Id was specifically transferred to naive rats with immune lymph node cells from cyclophosphamide-pretreated donors. When these donor cells were treated with anti-T cell antibodies (anti-RT7.1) and complement, however, this antiidiotypic reaction did not occur, suggesting the DTH reaction was T cell mediated. It was also of interest to observe in Table I that rats pretreated with low-dose cyclophosphamide, the same rats who made an antiidiotypic response to RE-Id, developed worse disease than rats immunized in a normal fashion ($P < 0.04$). This finding is consistent with the elimination of a cyclophosphamide-sensitive suppressor cell mechanism in rats with evolving nephritic lesions (9). A detectable antiidiotypic immune response to RE-Id in the setting of worse disease is also compatible with the view that a widespread loss of cyclophosphamide-sensitive suppressor function might obviate any subsequent suppressive antiidiotypic effect on disease.

Development of Suppressor T Cells Early in the Nephritogenic Immune Response. Rats normally immunized to produce disease also develop suppressor T cells during the afferent phase of the nephritogenic immune response (1), an effect not unlike that which we have previously observed in nephritic guinea pigs (7). Because rats with nephritis do not seem to make an antiidiotypic immune response unless they are pretreated with low-dose cyclophosphamide, we wished to determine if this suppressor cell effect might influence the development of antiidiotypic immunity. Before this question could be directly examined, however, we wanted to further characterize this suppressor cell system. In Fig. 3*A*, it can be observed that the lymphocyte proliferative response to Con A and PPD in nephritic rats, expressed as a percent of control, is maximally reduced ~2 wk after immunization. Over the same time period, the tubular antigen-reactive immune response in nephritic animals begins to rise and is fully established after

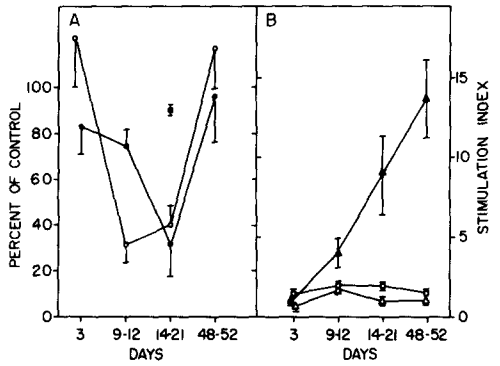

FIGURE 3. Interval comparisons of lymphocyte proliferative responses from nephritic and control rats to antigens and mitogens. (*A*) The blastogenic response to PPD (○) and Con A (●) of lymphocytes from nephritic rats expressed as a percent of control was suppressed as early as 9 d after immunization (four to six rats per group). The suppression was persistent for at least another 10–12 d. (■) compares the Con A response of CFA-immunized rats to normal; (*B*) In spite of the ongoing suppression, nephritic rats showed a strong proliferative response to SRTA (▲) compared with controls (□). The response of nephritic rats is tubular antigen-specific, as they did not respond to SLA (△).

TABLE III

A Representative Experiment Illustrating the Blastogenic Response to Antigens and Mitogens

Group*	Stimulant	[^{3}H]TdR incorporation‡			
		Con A	PPD	SRTA	SLA
RTA/CFA	+	9,727 (17.1)§	7,448 (7.5)	17,825 (17.9)	1,190 (1.2)
	−	567	992	992	992
CFA	+	45,969 (71.3)	22,399 (26.0)	980 (1.1)	667 (0.8)
	−	645	861	861	861

* Pooled lymph node cells from three animals per group harvested 14 d after immunization and cultured in the presence of mitogen or antigen as described in Material and Methods. Con A cultures harvested at 72 h; antigen cultures harvested at 96 h.

‡ Mean cpm for replicate cultures.

§ Stimulation index.

3 wk when compared with controls (Fig. 3*B*). The response to tubular antigen (SRTA) is specific as there is virtually no response to liver antigen (SLA) controls. The tabulated results of a representative experiment done with lymph node cells on day 14 can be seen in Table III. As illustrated in this experiment, both the PPD and Con A responses were markedly lower in the immune rats receiving RTA/CFA compared with an appropriate control.

We attempted to identify the cells responsible for this suppressive effect using a Con A-driven suppressor cell assay. In this assay, the ability of neutral responder cells to proliferate in the presence of Con A and irradiated lymph node cells from either nephritic or control animals can be compared and expressed as a percent suppression (7). In Table IV, the characterization of these suppressor cells was examined at several different doses of Con A. The findings at all three doses of Con A were remarkably similar, although the magnitude of the stimulation indices did vary with the amount of mitogen. The findings in this table

TABLE IV
Con A-stimulated Suppressor Cell Assay

Admixed cells*	Con A-induced stimulation index of neutral responder cells‡				
	10 μg/ml	10 μg/ml	5 μg/ml	5 μg/ml	2.5 μg/ml
RTA/CFA	6.3 ± 0.7 (96.8)§	58.3 ± 6.6 (31.4)	84.7 ± 9.5 (68.3)	141.9 ± 7.0 (56.4)	505.1 ± 10.3 (66.3)
RTA/CFA (TD)		72.4 ± 5.6 (4.3)		283.1 ± 12.4 (4.0)	1,265.3 ± 48.5 (0.1)
CTX-RTA/CFA	142.7 ± 1.5 (27.6)		198.4 ± 15.7 (25.8)		
Control (C')		75.7 ± 5.9		294.9 ± 34.0	1,274.9 ± 85.7
Control	197.1 ± 44.6	85.1 ± 11.6	267.6 ± 11.9	326.1 ± 38.0	1,388.1 ± 166.1

* The admixed lymph node cells were harvested from rats 14 d after immunization. These cells were treated with 3,000 rad before their use. TD, T cell depletion with anti-RT7.1 antibodies plus complement; C', cells treated with complement alone; CTX-RTA/CFA, donor animals were pretreated with cyclophosphamide before receiving RTA/CFA.

‡ The admixture of neutral responder and irradiated cells was cultured at a 1:1 ratio in various concentrations of Con A for 72 h. Data is expressed as mean stimulation index ± SEM.

§ Numbers in parentheses equal the percent suppression compared with control.

suggest that immune lymph node cells harvested from RTA/CFA rats on day 14 markedly suppressed the Con A response of neutral responder cells. This suppression was not nearly as evident if the RTA/CFA co-cultured cells were from cyclophosphamide-pretreated donors, suggesting the suppressor cell was sensitive to cyclophosphamide. The suppression observed in the presence of irradiated lymph node cells from nephritic rats was also abrogated when these cells were pretreated with anti-RT7.1 antibodies and complement. From the data collected in these experiments it would seem that, in addition to developing disease, nephritic rats also develop a nonspecific suppression that occurs early in the immune response, is T cell mediated, and cyclophosphamide sensitive.

Effect of Nonspecific Suppression on the Development of Antiidiotypic Immunity. Because nephritic rats do not normally make an antiidiotypic immune response towards polymorphic αTBM-Ab, we wished to assess the potential effect of this nonspecific suppression on the development of antiidiotypic immunity. To perform these experiments, we took advantage of methods that allow the measurement of DTH responses to idiotype using a footpad assay (19). In our experiments, naive rats were subcutaneously immunized with idiotype expressed on monoclonal αTBM-Ab BN52/19-coated spleen cells. The immunized animals were challenged 5–6 d later with 50 μg of BN52/19 in PBS. The other hind footpad was usually injected with PBS alone, although injecting with PBS or leaving the footpad uninjected did not seem to make very much difference. In Fig. 4, rats immunized with BN52/19-coated spleen cells, who were then challenged with BN52/19, developed significant, incremental footpad swelling when compared with animals not immunized but challenged with the same dose of BN52/19. The fine specificity of this DTH reaction was demonstrated by immunization with control cells (EDCI alone), or by challenge with purified IgG or BN48/12 monoclonal αTBM-Ab, which also did not induce significant footpad swelling. Rats were also injected with HGG-coated spleen cells to establish the fact that the response to BN52/19 was not unique to this antibody. Rats immunized with HGG-coated spleen cells made a significant DTH response to HGG compared with control, but similarly immunized animals did not respond when challenged with BN52/19. In Table V, we also observed that the DTH

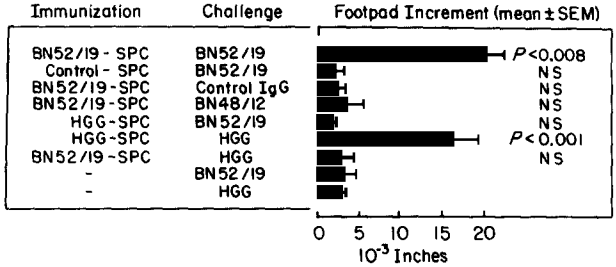

FIGURE 4. DTH response to idiotypes expressed on BN52/19. Rats were subcutaneously immunized with monoclonal αTBM-Ab BN52/19-coated spleen cells (SPC). 5–6 d later, one footpad was challenged with BN52/19, and a significant increment in swelling occurred compared with the PBS-challenged control footpad. If rats were immunized with control cells (EDCI-treated) and challenged with BN52/19, no response was demonstrated. The reaction was specific for idiotype because rats immunized with BN52/19-SPC did not respond to control IgG, BN48/12, or HGG. If the immunization was done with HGG-spleen cell, a specific response could also be demonstrated to HGG (four to six rats per group were used).

TABLE V

Transfer of DTH to Monoclonal αTBM-Ab

Donor cells*	*n*	Treatment	BN52/19 DTH response‡
BN52/19 immune	4	—	15.0 ± 0.8§
BN52/19 immune	7	C′	13.4 ± 0.9§
BN52/19 immune	4	αRT7.1 + C′	2.8 ± 1.8
Control	5	—	4.4 ± 0.5
—	4	—	3.8 ± 0.5

* Donor lymph node cells harvested from rats 5 d after immunization with BN52/19-coated spleen cells were transferred into naive recipients.
‡ Recipients were challenged 4–6 h after transfer and the mean footpad increment in inches times 10^{-3} ± SEM was determined 24 h later.
§ $P < 0.001$ compared with unimmunized controls.

response to BN52/19 could be adoptively transferred to a naive rat with BN52/19-reactive cells. Such a transfer, however, was inhibited if the donor cells were pretreated with anti-RT7.1 antibody and complement, suggesting that the DTH response to BN52/19 was T cell mediated. Selected footpads were also evaluated histologically, and were found to contain mononuclear infiltrates, particularly around blood vessels (data not shown).

With this footpad assay, we then examined the influence of transferred lymphocytes from nephritic rats on the development of antiidiotypic immunity to BN52/19. In Fig. 5 (group I), it can be observed that lymph node or spleen cells from nephritic rats could markedly inhibit the development of antiidiotypic immunity when they were transferred into naive animals at the time of their immunization with BN52/19-coated spleen cells. This suppressive effect could be abolished by T cell depletion, as well as when the transferred cells were harvested from cyclophosphamide-pretreated donors. Furthermore, lymph node or spleen suppressor cells could attenuate a similar DTH response to HGG-coated spleen cells (See Fig. 5; group II). In Table VI, the suppressive effect of NWT cells from nephritic rats was compared with the effect of NWA cells. By indirect fluorescence, the NWT population from lymph nodes contained <5%

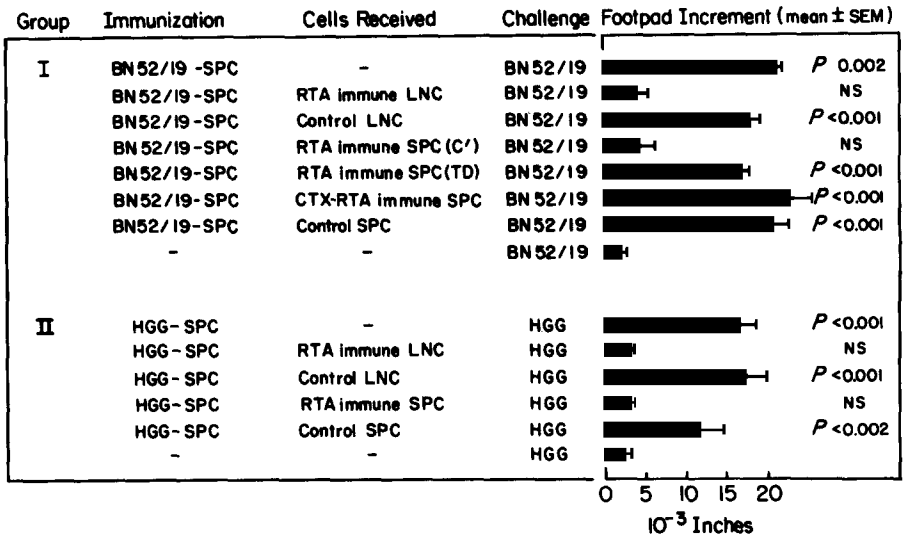

FIGURE 5. Assessment of a suppressor T cell effect from nephritic rats on the development of antiidiotypic immunity. In group I, all rats (four to eight rats per group) were immunized with BN52/19-SPC (SPC, spleen cells; LNC, lymph node cell). Other lymphocytes from nephritic or control rats were intravenously administered at the time of immunization. 5–6 d later each group was challenged with BN52/19. Immune spleen or lymph node cells from nephritic rats prevented the development of an antiidiotypic immune response. Such inhibition was averted if the lymphocytes were T cell-depleted (TD) before transfer, or if they were derived from cyclophosphamide (CTX)-pretreated donors. In group II, suppression was also provided by lymphocytes from nephritic rats such that a DTH response was not made to HGG. This latter finding suggests that the overall suppressive effect was nonspecific.

TABLE VI

Suppression of Antiidiotypic Immunity with OX8⁻ T Lymphocytes

Immunization*	*n*	Cells transferred‡	Cell dose	BN52/19 DTH response§
BN52/19-SPC	5	—	—	17.0 ± 0.4‖
BN52/19-SPC	4	RTA/CFA	5×10^7	7.0 ± 0.7
BN52/19-SPC	3	RTA/CFA (NWT)	5×10^7	6.3 ± 0.8
BN52/19-SPC	3	RTA/CFA (NWA)	5×10^7	15.5 ± 0.5‖
BN52/19-SPC	5	RTA/CFA (OX8⁻)	1.2×10^7	6.2 ± 0.4
BN52/19-SPC	4	RTA/CFA (OX8⁺)	1.2×10^7	14.8 ± 0.9‖
BN52/19-SPC	4	CFA	5×10^7	15.5 ± 0.5‖
—	4	—	—	3.8 ± 0.5

* Rats received 5×10^7 derivatized cells subcutaneously. SPC, spleen cells.

‡ At the time of immunization, groups of rats also received pooled spleen/lymph node cells: RTA/CFA and CFA cells were harvested from other rats 14 d after immunization; OX8⁻, NWT that do not bind OX8 antibodies; OX8⁺, NWT that bind OX8 antibodies.

§ Recipients were challenged with BN52/19 at 5 d after immunization and the mean footpad increment in inches ± SEM was determined 24 h later.

‖ $P < 0.001$ compared with unimmunized controls.

Ig⁺ cells. Of these T cells, ~15–17% were OX8⁺. These NWT were further separated into OX8⁻ or OX8⁺ subpopulations by an indirect panning technique. After separation on panning dishes, the nonadherent cells were 93% OX8⁻ and the adherent cells were 86% OX8⁺. The results shown in Table VI suggest that

the suppressive effect in the lymph nodes of nephritic rats is T cell mediated by an $OX8^-$ cell. Interestingly, the effect was not demonstrated by $OX8^+$ cells alone.

Inhibition of Disease by the Adoptive Transfer of Nonspecific Suppressor Cells or by the Transfer of Idiotype-reactive Cells. The studies performed thus far demonstrate that rats normally immunized to produce disease do not develop an antiidiotypic immune response towards idiotypes expressed on αTBM-Ab. The failure to express this antiidiotypic immunity may be partly due to the concomitant development of an $RT7.1^+$, $OX8^-$ suppressor T cell. If rats are pretreated with cyclophosphamide, however, this suppressor T cell does not appear, antiidiotypic immunity can be demonstrated, and disease is much worse (see Table I). The reason that disease is much worse in cyclophosphamide-pretreated rats may be related to the fact that low-dose cyclophosphamide not only removes the nonspecific suppressor cell, but also removes the suppressive limb of the antiidiotypic immune response. We were interested to know what the effects of such an antiidiotypic immunity might be, were it allowed to develop. To test these proposals further, we adoptively transferred immune cells, with or without cyclophosphamide pretreatment, into naive recipients. The recipients were rested for 5 d and then immunized with or without cyclophosphamide pretreatment. We reasoned that, in such a protocol, the transfer of idiotype-reactive cells (see Table I) unencumbered by nonspecific suppression might induce a suppressive antiidiotypic effect (22, 23). In Table VII, this hypothesis was tested using nine different experimental groups. It should be pointed out first, however, that immune lymphocytes from nephritic rats, under a variety of experimental conditions, do not transfer disease when they are intravenously injected into

TABLE VII
Adoptive Transfer of Antiidiotypic Immunity

Group	Donor cells*	Immunization‡	DTH response§		Renal histology‖
			SRTA	RE-Id	
1	—	RTA/CFA	22.5 ± 0.6**	3.1 ± 0.4	1.8 ± 0.2
2	CTX-RTA/CFA¶	CTX-RTA/CFA	28.0 ± 2.7**	16.0 ± 1.4**	2.1 ± 0.6
3	CTX-RTA/CFA	RTA/CFA	7.5 ± 0.7‡‡	15.8 ± 0.9**	0.1 ± 0.1
4	CTX-RTA/CFA	CFA	14.3 ± 1.2**	2.6 ± 0.3	0.0 ± 0.0
5	RTA/CFA	RTA/CFA	8.0 ± 0.4‡‡	3.5 ± 0.6	0.1 ± 0.1
6	RTA/CFA	CFA	9.0 ± 1.5‡‡	2.3 ± 0.6	0.0 ± 0.0
7	CFA	RTA/CFA	21.5 ± 1.3**	1.5 ± 0.5	1.0 ± 0.0
8	CFA	CFA	2.6 ± 1.2	2.6 ± 0.3	0.0 ± 0.0
9	—	—	2.8 ± 0.3	2.5 ± 0.5	—

* Donor lymph node cells harvested from rats 14 d after immunization were transferred into naive recipients.

‡ The recipients (three to four rats per group) were immunized as indicated 5 d after these cell transfers.

§ Data points were calculated as a mean footpad increment in inches times 10^{-3} ± SEM, 22 d after immunization, 24 h after challenge.

‖ Severity of the interstitial lesions was qualitatively graded from 0 to 4 and expressed as a mean ± SEM.

¶ 50 mg/kg of cyclophosphamide (CTX) was administered 3 d before immunization.

** $P < 0.001$ compared with unimmunized controls.

‡‡ $P < 0.05$ compared with unimmunized controls.

naive recipients (24; Zakheim and Neilson, unpublished observations). Group 6 confirms our previous experience that disease is not transferred with immune cells. In group 5, compared with group 7, the immunization with RTA/CFA also did not induce disease after the transfer of immune lymphocytes. We have observed a similar finding in guinea pigs, and have attributed the lack of disease to the inhibitory influence of nonspecific suppressor T cells in the transferred inoculum (Neilson et al., manuscript submitted). Furthermore, the rats in group 5 made no DTH response to RE-Id, and only a mild one to SRTA. In contrast, when donor cells from cyclophosphamide-pretreated rats (group 3) were given to recipients who were then immunized with RTA/CFA, a significant antiidiotypic immune response was made to RE-Id. Only a very small response, however, was made to SRTA, and no disease was observed, suggesting that the transfer of RE-Id-reactive cells before the induction of disease could suppress the later development of lesions. In Table II, we demonstrated that the expression of this antiidiotypic effect is T cell mediated. In Table VII (group 2), however, if the recipients receiving the same cells as group 3 were treated with cyclophosphamide before their immunization with RTA/CFA, the antiidiotypic immune response appeared, but so did disease. These findings also suggest that low-dose cyclophosphamide may impair the suppressive phase of antiidiotypic immunity. Comparing group 4 with groups 2 and 3, it is furthermore evident that the antiidiotypic immune response to RE-Id is only demonstrable if the recipients are immunized with RTA/CFA. Finally, we would propose without formal proof, that the greater intensity of disease in group 1 compared with group 7 might reflect a reduced effect of the adjuvant in rats receiving CFA-reactive cells, a condition that is equally shared by all experimental groups except group 1.

Discussion

The present studies illustrate several interesting findings related to the antiidiotypic immune response in experimental interstitial nephritis. One unexpected observation made during the initial preparation of the positive control antiidiotypic antibodies was that some degree of serologic cross-reactivity could be demonstrated between TB-αId and RE-αId antisera. This finding, however, must be qualified on at least two counts. First, the observation was made with polymorphic antisera that possibly contained other unknown cross-reactivities. Second, the specificities of the antiidiotypic antibodies, during their derivation, were vulnerable to the potential source impurity of the idiotypes in question. It should be pointed out, however, that while the preparation of the TB-αId antisera could have been contaminated by a small number of B cells, it is unlikely that the preparation of the RE-αId antisera was contaminated by the presence of T lymphocytes. Preliminary experiments furthermore suggest that RE-αId antibodies selectively bind highly purified T cells from immune rats with interstitial nephritis (data not shown). Thus, despite the above qualifications, the observed cross-reactivity suggest there might be shared idiotypic determinants between the T and B cell repertoires producing αTBM disease, a finding supported by previous studies in other experimental systems (25, 26).

One of the principle aims of the present experiments, however, was to determine if rats normally immunized to produce disease made any regulatory

or protective antiidiotypic immune response. Previous experiments suggest that rats pretreated with antigen-reactive T lymphoblasts (6) or RE-αId antisera (27) do not develop αTBM disease because of an antiidiotypic effect. RE-αId antisera can also block the development of interstitial nephritis in guinea pigs (28). In screening radioimmunoassays using RE-Id and BN52/19, we were nevertheless unable to demonstrate any binding reactivity to these antibodies using interval sera from rats with nephritis. Nor could any cell-mediated antiidiotypic immune response be demonstrated using a measured DTH reaction to RE-Id. The failure to observe a regulatory antiidiotypic effect is also consistent with our unpublished observation that αTBM-Ab titers to collagenase-solubilized tubular antigen peak around day 32, but do not appreciably fall over an ensuring interval of 68 d. Interestingly enough, however, both a humoral and cell-mediated antiidiotypic immune response could be observed if the rats were pretreated with low-dose cyclophosphamide before immunization. Furthermore, the cell-mediated antiidiotypic response could be adoptively transferred by T lymphocytes.

The finding that an antiidiotypic immune response appeared after pretreatment with cyclophosphamide provided an opportunity to both determine the effect of this antiidiotypic immunity on the expression of disease, and to characterize the suppressor mechanism that normally inhibits its development. From the information contained in the present studies, it seems that shortly after the induction of disease, a cyclophosphamide-sensitive, nylon wool nonadherent, $RT7.1^+$, $OX8^-$ suppressor T cell appears. The effect of this suppressor cell system is nonspecific; it can inhibit the proliferation of immune lymphocytes to mitogens or antigens, and can inhibit the development of an antiidiotypic immune response to monoclonal αTBM-Ab. While the appearance of the suppressor T cell system is tubular antigen dependent, it is not yet known whether the effect is genetically restricted (29). The comparative observation that an SRTA response normally develops after immunization despite the subsequent generation of nonspecific suppression, whereas an antiidiotypic effect does not, is we believe, related to kinetic factors. That is, the latent development of nonspecific suppressor cells can only blunt, but not totally block an emerging SRTA response, while the operational presence of this suppression at the time of potential induction of an antiidiotypic effect, is completely inhibitory. The timing of this nonspecific suppression, 7–21 d after immunization, furthermore seems to permanently hamper the development of an antiidiotypic effect during the course of disease. The observation in adoptive transfer experiments that if suppression is present at the time of immunization, disease does not occur (Table VII), and the observation that cyclophosphamide pretreatment of immune animals produces worse disease (Table I), each suggest that this suppressor cell mechanism can directly influence both the induction and effector phase immune responses, a feature of some nonspecific suppressor systems (30). The use of low-dose cyclophosphamide has been associated with a selective depletion of suppressor T cells in mice (31–33) and the enhancement of experimental arthritis in rats (9). Consistent with other studies (31), we did not observe any significant decrease in the αTBM-Ab antibody titers of nephritic rats that were pretreated with 50 mg/kg of cyclophosphamide (data not shown).

Nonspecific suppressor T cells that have been studied in mice after immuni-

zation with antigens or haptens are reported to be cyclophosphamide sensitive and either Lyt $1,2,3^+$ (34) or Lyt $1^+,2^-$ (30) cells. Con A-induced nonspecific suppressor T cells, conversely, seem to be Lyt $1^-,2^+$ (35). The suppressor T cell we have identified in the present study in rats is $OX8^-$, not the classical $OX8^+$ suppressor cell phenotype. Rather, it appears to be phenotypically similar to the suppressor cells described by Asano and Hodes (29, 30). The nonspecific suppressor cells in their experiments were Lyt $1^+,2^-$, but required the participation of unprimed Lyt 2^+ T cells to complete the suppressive effect. In our experiments, $OX8^+$ T cells from nephritic rats, alone, could not inhibit the development of antiidiotypic immunity. We do not know as yet, however, whether $OX8^+$ cells are required for the $OX8^-$ effect. While not formally established, the nonspecific suppressor T cell we have observed in the present experiments may also represent a subpopulation of suppressor-inducer cells (23, 36). Why nonspecific suppression should occur in this model of renal disease is not entirely clear. Its development precedes a deterioration in renal function so that the nonspecific effects of uremia do not seem applicable. The supressive effect is also completely eliminated by T cell depletion, and can be transferred with nylon wool T cells, suggesting that an additional nonspecific macrophage effect is unlikely. The appearance of nonspecific T cell suppression may reflect a feedback response to a broad variety of non- or peri-antigen-specific immune responses made early in the process of tubular antigen recognition (37). In support of this hypothesis, it has been known for many years that a large percentage of the immunoglobulin produced shortly after immunization is not specific for the primary antigen (38). This background activity disappears after several weeks whereas specific antibody to the antigen remains (39). Such nonspecific activity may be related to the purity of the antigen, its dose, or the strength of the adjuvant (40). It may also be the result of an amplified T helper cell response that selects the relevant isotypes and idiotypes that form the final effector repertoire (41, 42).

The detection of an antiidiotypic immune response in the setting of worse disease (Table I) after pretreatment with cyclophosphamide is consistent with the view that a widespread loss of cyclophosphamide-sensitive suppressor cell function might limit or obviate any subsequent suppressive antiidiotypic effect on disease. To test this point further, we performed a series of adoptive transfer experiments (Table VII). In the design of these experiments, the cotransfer of contaminating CFA-reactive cells potentially reduced the overall magnitude of disease in all groups subsequently immunized with RTA in CFA. The presence of such cells probably induced an inhibitory effect towards the adjuvant. Despite this dampening, however, we did observe that when immune lymphocytes from cyclophosphamide-pretreated donors were transferred into naive recipients that were later immunized to produce disease, an antiidiotypic immune response appeared and disease did not develop. This finding demonstrates that idiotype-reactive lymphocytes (perhaps T cells; see Table II), unencumbered by nonspecific suppressor cells, seem to induce a suppressive antiidiotypic effect on the development of disease.

Summary

Antiidiotypic immunity can successfully inhibit the development of anti–tubular basement membrane (αTBM) disease that produces interstitial nephritis. Rats normally immunized to produce disease, however, do not develop this

regulatory and protective antiidiotypic effect. The failure to see such a regulatory response is functionally related to the influence of a nonspecific, $RT7.1^+$, $OX8^-$ suppressor T cell that appears shortly after immunization. While this suppressor cell system can partially reduce the intensity of disease, it also limits the host's ability to specifically regulate the αTBM immune response and, hypothetically, leaves the disease process in an operationally active mode.

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