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Organ-Specific Disease Provoked by Systemic Autoimmunity

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Summary

Rheumatoid arthritis (RA) is a chronic joint disease characterized by leukocyte invasion and synoviocyte activation followed by cartilage and bone destruction. Its etiology and pathogenesis are poorly understood. We describe a spontaneous mouse model of this syndrome, generated fortuitously by crossing a T cell receptor (TCR) transgenic line with the NOD strain. All offspring develop a joint disease highly reminiscent of RA in man. The trigger for the murine disorder is chance recognition of a NOD-derived major histocompatibility complex (MHC) class II molecule by the transgenic TCR; progression to arthritis involves CD4+ T, B, and probably myeloid cells. Thus, a joint-specific disease need not arise from response to a joint-specific antigen but can be precipitated by a breakdown in general mechanisms of self-tolerance resulting in systemic self-reactivity. We suggest that human RA develops by an analogous mechanism.

Introduction

Rheumatoid arthritis is a chronic inflammatory disease of the synovial joints (Feldmann et al., 1996a, 1996b). A mixed population of leukocytes invades the synovial membrane and fluid, both normally devoid of blood-derived cells; concomitantly, resident synovial macrophages and fibroblasts become activated and divide. The different leukocyte and synoviocyte constituents produce a complex melange of cytokines and other soluble mediators, which are thought to be responsible, directly or indirectly, for the characteristic neovascularization, cartilage destruction, and bone erosion. Systemic manifestations may ensue, including elevated titers of autoantibodies in the blood, vasculitis, or abnormalities of other organs, e.g. the lung.

RA is an important disease, affecting a significant portion (~1%) of the population and inflicting substantial pain and disability. It is also a mysterious disease. We still do not know whether it is primarily autoimmune or inflammatory in nature, whether there is an inciting infectious agent, self-antigen, or both, to what extent initiation and progression depend on systemic versus joint-specific events, and whether the primary effectors are T cells, B cells, or other leukocytes. Our uncertainty is compounded by two factors: diagnosis of RA is usually made only after the disorder has progressed rather far along its course, probably long after the inciting events; and RA appears to be a very heterogeneous entity, with significant variation in age of onset, overall severity, and pathology.

Clearly, attempts to understand and control rheumatoid arthritis would benefit greatly from a small animal model that spontaneously and reproducibly develops a resemblant disease. Here we describe a new mouse model of RA, generated fortuitously, and explore the mechanism of pathogenesis.

Results

Generation of the KRN Transgenic Mouse Line

R28 is a T cell hybridoma that was derived from a B10A.4R mouse and recognizes the 41–61 peptide of bovine pancreas ribonuclease (RNase) in the context of A^k (Peccoud et al., 1990). To study selection of the R28 specificity, we generated a transgenic mouse line carrying the rearranged T cell receptor genes from the hybridoma. TCR-α and -β cDNAs were synthesized from mRNA and were cloned and sequenced, revealing $V_\alpha 4$ and $V_\beta 6$ variable regions (S. Candéias, C. B., D. M., unpublished data). The variable segments were inserted into cassette genomic vectors containing homologous TCR transcription signals and known to direct efficient and specific expression in transgenic animals (Kouskoff et al., 1995). Large TCR-α and -β genomic fragments were coinjected into C57Bl/6(B6)xSJL F2 embryos, and a transgenic line, KRN, was established by repeatedly back-crossing a founder carrying both genes to the B6 strain.

The KRN line was also crossed with various strains harboring the H-2^k allele at the MHC in order to introduce the appropriate restriction element, A^k, presumably required for positive selection of the R28 specificity. The resulting offspring expressed both transgene-encoded TCR chains on a large proportion of T lymphocytes in the thymus and periphery, and a vigorous proliferative response could be elicited when their lymph node cells were challenged with the bovine RNase 41–61 peptide in the presence of A^k-expressing antigen presenting cells (APCs) (Kouskoff, 1994; data not shown). Curiously, though, the TCR transgenes did not promote skewing of T cells into the mature $CD4^+$ compartment of either the thymus or periphery of animals expressing A^k, as was expected from previous results in analogous TCR transgenic systems (Berg et al., 1989). In fact, by most measures of positive selection, the T cell compartments of animals on the "selecting" H-2^k and "nonselecting" H-2^b backgrounds were quite similar, suggesting poor

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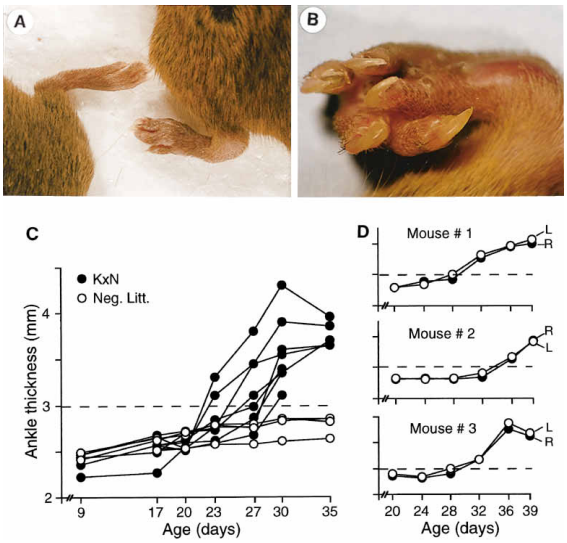


Figure 1. Joint Swelling and Deformation

(A) Hind limbs of 6-week-old transgene-positive and -negative KRNxNOD littermates.

(B) Toe deformation at three months.

(C) Ankle thickness followed over time in a cohort of KRNxNOD transgenics (KxN) and transgene-negative littermates (neg. litt.). Each solid line represents a single mouse. The dashed line shows the maximum value for control mice at these ages.

(D) Left (L) and right (R) ankles measured in parallel for three individuals.

selection of transgene-encoded TCRs in both cases, but some peripheral emergence of the R28 specificity, nonetheless, probably due to "piggy-backing" with receptors encoded by endogenous TCR genes that had bypassed allelic exclusion (Borgulya et al., 1992).

At that point, KRN did not appear to be a very useful line: transgene-encoded TCRs were expressed quite efficiently, but were ineffective at promoting allele-specific positive selection. Fortunately, and quite fortuitously, we also crossed KRN to the NOD strain.

Development of Arthritis in KRNxNOD Mice

When the KRN line was crossed with the NOD strain, a surprising phenotype appeared in the offspring: transgene-positive, but not transgene-negative, mice exhibited pronounced joint inflammation (compare Figure 1A right and left). In the transgene-positives, all of the distal joints of the paws were swollen and red. As the animals aged, a variety of deformations became evident—e.g., hyperextension of the ankle, valgus deviation of the knee, hyperpronation of the toes (Figure 1B)—and the animals' mobility was compromised, probably explaining a reduced reproductive performance. Otherwise, their health appeared normal (including kidney function and the quality of the skin and nails). Since this disease was ostensibly similar to rheumatoid arthritis, we undertook an extensive characterization, assessing a diversity of clinical, histological and immunological parameters.

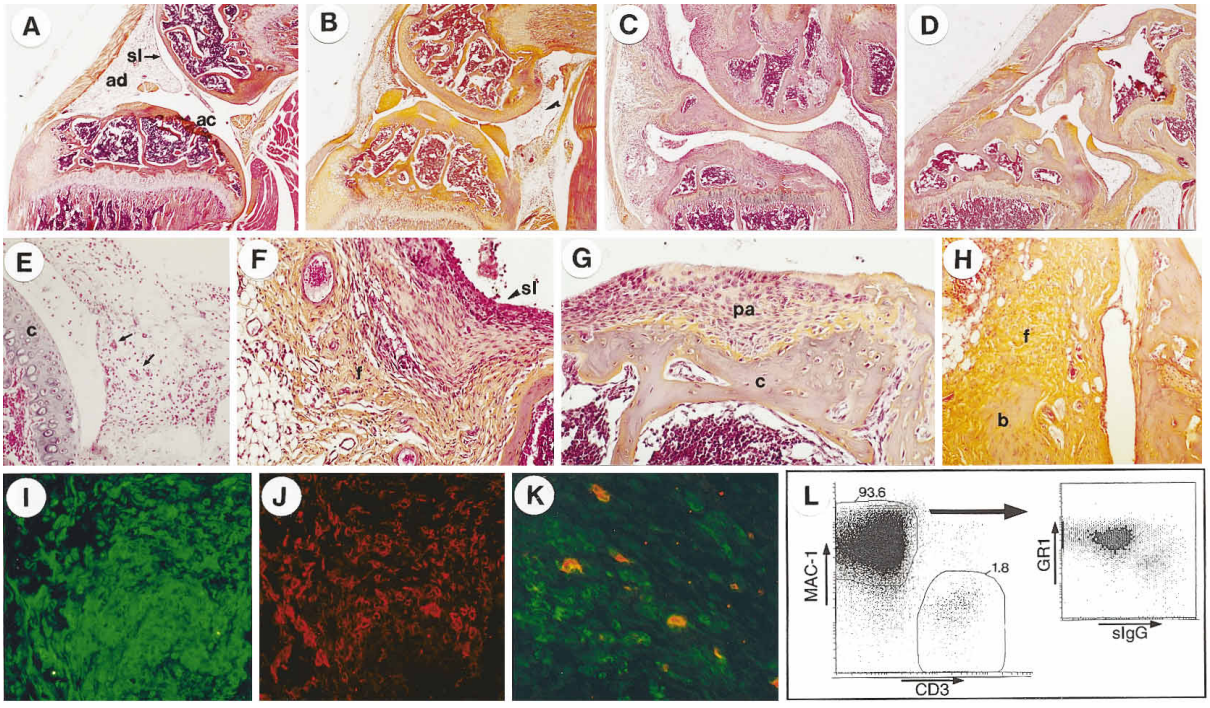


Figure 2. Histology of Arthritis

(A–D) Hematoxylin-eosin-safran staining of knee sections of a normal mouse (A) and KRNxNOD transgenics after 1 day (B), three weeks (C), or three months (D) of disease. ac, articular cavity; sl, synovial lining; ad, adipose tissue; arrow in (B), intraarticular cells and fibrinoid material.

(E) Hypervascularization and subsynovial infiltration at day 1. Arrows indicate vessels.

(F) Synovitis at 3 weeks. f, fibrosis.

(G) Pannus (pa) formation at 3 weeks. c, cartilage.

Clinical Onset

To quantitate disease onset, we measured ankle thickness in a cohort of transgene-positive and -negative littermates beginning shortly after birth. Figure 1C demonstrates a sharp onset of swelling at 25–35 days in the former but not the latter. This panel also illustrates the complete penetrance of the disease; indeed, in the hundreds of animals by now examined, we have observed joint inflammation in all of the KRNxNOD TCR transgenics and none of the transgene-negative littermates (also see below). This was true whether the mice were housed under specific-pathogen-free or conventional conditions.

Comparison of the curves for any two individuals in Figure 1C revealed a degree of heterogeneity in disease initiation and course. However, when curves for the two limbs of individual animals were compared (Figure 1D), they were always precisely superimposable. Symmetry of involvement also appeared true of the other inflamed joints, according to simple by-eye inspection.

Joint Histology

To visualize the pathological processes underlying these clinical manifestations, we performed histological analysis of knee joints (Figure 2). In the joint from a normal mouse (Figure 2A), the articular cavity was acellular, the synovial lining a thin unicellular layer covering adipose and connective tissue, and the cartilage smooth and uniform. At disease onset in KRNxNOD mice (Figure 2B), the lesions were quite discrete: fibrinoid material and a few cells were found in the cavity; edema set in under the synovial lining, accompanied by neovascularization and some infiltration of inflammatory cells (seen better at higher power in Figure 2E); and bone and cartilage were largely unaffected. After a few weeks of evolution (Figure 2C), disease was marked by extensive synovitis, affecting all areas of the joint. The synovium was hyperplastic, with massive infiltration of inflammatory cells and the beginnings of fibrosis (Figure 2F). In some areas, small nodules developed, seemingly composed of lymphocytes. Abundant fibrinoid material and inflammatory cells, mainly neutrophils in appearance, exuded from the inflamed synovium into the articular cavity. The synovitis extended over some areas of cartilage, forming a typical pannus, which invaded the cartilage and underlying bone; chondrocytes in their niches were pyknotic (Figure 2G). After several months of disease, the knee joint presented an anarchic picture (Figure 2D). The inflammatory process had largely receded, but the architecture of the joint was completely remodeled: little cartilage remained and the residual bone adopted irregular structures, embedded in the massive fibrosis that now made up most of the joint (Figure 2H).

Similar lesions were detected in the distal joints of the limbs of all KRNxNOD mice examined—including the metatarsal, tarsal, ankle and knee joints, and the corresponding forelimb articulations. Interestingly, the hip joint was unaffected, even in animals whose knees were completely destroyed. Inflammation of the spine was noted in some regions of some animals; however, it differed markedly from the typical limb joint inflammation in its mild and variable appearance.

Fluorescent antibody staining of cryostat sections of the knee joint revealed that the most abundant cell types were of the macrophage lineage, identified by their staining with the MAC-1 and MOMA-1 markers (Figures 2I and 2J). T lymphocytes were less frequent, even at the earliest stages of disease, and were usually found in small clusters (Figure 2K); B cells were even rarer (not shown). Cytofluorimetric analysis of ankle synovial fluid showed a somewhat different composition in the articular cavity (Figure 2L). There was a minor population of small $CD3^+$ T lymphocytes (1%–3%) and a vast majority of large Mac-1-positive cells. Most of these were neutrophils, identified by their scatter profiles and strong staining with an anti-GR-1 mAb, but their was also a small component of GR-$1^{+/-}$, surface immunoglobulin $(sIg)^+$ cells. The paucity of lymphocytes in the joint was reflected in the profile of cytokines detected—by PCR analyses of cells in the synovial fluid: undetectable interleukin (IL)-4, barely measurable IL-2 and interferon (IFN)-γ, and high levels of tumor necrosis factor (TNF)-α; by ELISA assays of protein in the synovial liquid: undetectable IL-4, IL-10, IFN-γ, TNF-α, and granulocyte/macrophage-colony stimulating factor (GM-CSF), and enormous quantities of IL-6 (0.5–20 μg/ml) (not shown).

Skeletal preparations were made from animals three to six months after disease onset to better document the distribution and extent of bone destruction (Figure 3). Damage was massive in the paws (Figure 3A), affecting all interphalangial, metatarsal, and tarsal joints (and their forelimb equivalents); the calcaneum was also damaged. A close-up view of the interphalangial joints (Figure 3B) depicts a typical image of bone erosion together with anarchic reconstruction; very similar pictures were found with the knee joint (Figure 3C). In contrast, the sacro-iliac joint always appeared intact (Figure 3D), as did the hip (Figure 3E). Consistent with the histological findings described above, most of the vertebral joints were unaffected, but signs of destruction were detected sporadically (arrow in Figure 3F).

Immunological Analysis

Lastly, we examined immunological parameters affected in certain of the human arthritides. Splenomegaly was often noted, and B lymphocyte numbers were increased in the lymph nodes and spleen of diseased animals (on average 1.75-fold; $n = 7$). However, B cells did not appear to be in an abnormal activation state as assessed by size, IgM/IgD profile, or staining intensity with anti-CD69, -CD5, or -MHC class II reagents (not shown). In addition, the follicular organization (follicle, mantle zone, germinal centers) appeared entirely normal. Hypergammaglobulinemia was also evident, essentially due to an increase in the IgG1 isotype (Figure 4A).

(H) Residual fibrosis (f) after 3 months. b, bone.

(I) Cryostat section of a knee synovium after one week of disease, stained with anti-MAC-1.

(J) As in (I), stained with anti-MOMA-1.

(K) As in (I), stained with anti-MAC-1 (green) and -CD3 (red).

(L) Cytofluorimetric analysis of synovial fluid cells. Inset, anti-GR1 versus anti-IgG staining on MAC-1^+CD3^- gated cells.

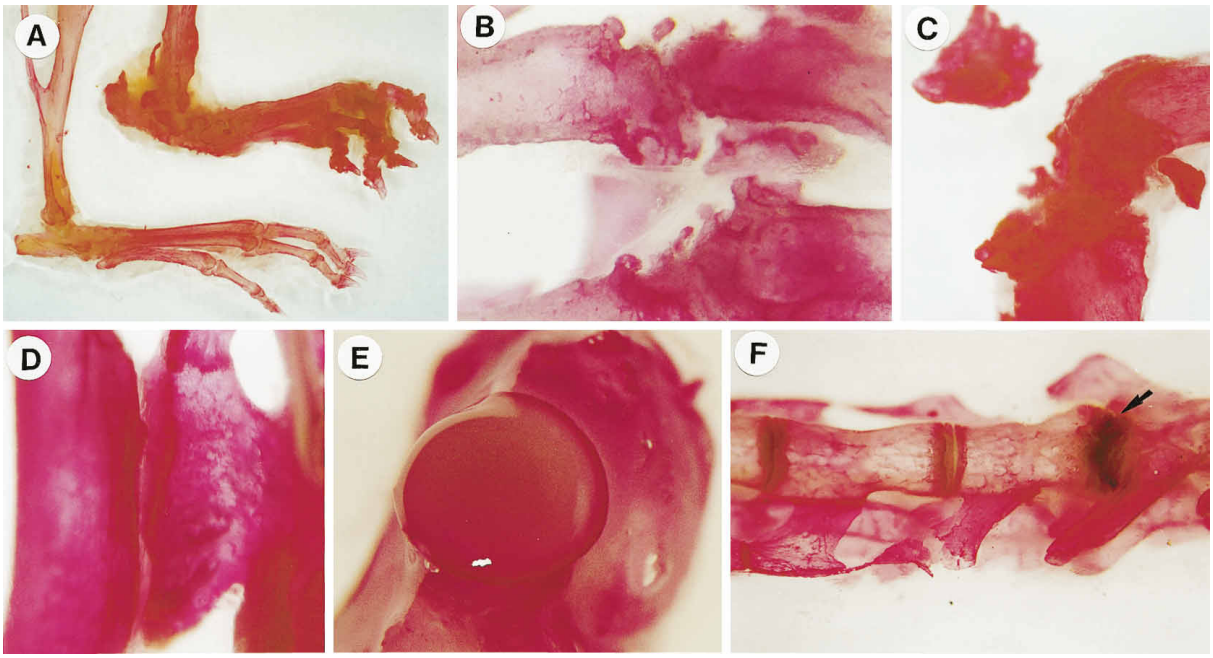


Figure 3. Selective Joint Destruction

Skeletal preparations after 5 months of disease. Extensive destruction in the paw compared with a negative littermate (A). Better visualized at higher power on an interphalangial joint (B), and in the knee (C). Intact sacro-iliac (D) and hip (E) joints. Some intervertebral joints (F) were affected (arrow), others largely intact.

Nonetheless, circulating antoantibodies could not be detected—including anti-dsDNA or rheumatoid factor (RF; IgM anti-IgG) (Figure 4B) and antibodies capable of binding to sections of liver or kidney (not shown). However, vast IgG deposits were observed on sections of many organs, mainly along the basal membranes, in a pattern that is distinctly different from that observed in diseases due to immune complex deposition, e.g. in MRL/lpr or NZBxNZW mice (Figures 4C–4G). IgG deposits were first seen some weeks after the initial signs of arthritis and were not associated with any other histological abnormalities.

As will be discussed in detail below, the overall image of disease in KRNxNOD mice—synthesized from diverse clinical, histological and immunological features—is highly reminiscent of (though not identical to) rheumatoid arthritis in human patients. Thus, although it was generated entirely unintentionally, we saw this as a potentially powerful model and have focused on unravelling disease etiology and pathology.

Genetic Contributions to Disease—the NOD-Derived A_β^{g7} Gene Is Primordial

An important clue to the etiology of arthritis in the KRNxNOD model came from genetic analysis. As mentioned above and further detailed in Table 1, the TCR transgenes did not engender pathology when carried on a B6 genetic background. Disease was first manifest in transgene-positive F1 offspring of a B6xNOD cross and was universal in the hundreds of animals so far analyzed. In contrast, transgene-positive F1 offspring from crosses between B6 and other inbred strains (BALB/c, DBA/2) showed no overt pathology.

The NOD strain is prone to autoimmune disease—most notably diabetes, but also sialitis, thyroiditis, and others (Tisch and McDevitt, 1996). Both its peculiar MHC allele, H-2^{g7}, and multiple non-MHC genes play a role in promoting autoimmunity (Wicker et al., 1995). To distinguish the contribution of MHC from non-MHC NOD-derived genes in our arthritis model, we crossed KRN with a B6 line congenic for the NOD MHC. The offspring (B6xB6.H-2^{g7}) had one MHC allele originating from the B6 and one from the NOD strain; all non-MHC genes were B6-derived. These animals all showed overt signs of arthritis (Table 1), indicating that the only required contribution from the NOD genome was one H-2^{g7} allele.

The NOD MHC is a complex recombinant haplotype, carrying d-alleles on the H-2K end, an unusual allele (g7) at the A_β locus, and b-alleles on the H-2D end. That neither B6xBALB/c nor straight B6 transgene-positive mice showed signs of pathology suggested that K^d, A_α^d (identical to A_α^{g7}) and Db were not sufficient in and of themselves to engender disease. To evaluate the importance of A_β^{g7}, we introduced an additional transgene, a cDNA expression construct promoting display of A_β^{g7} on all the usual class II–positive cells; since the transgene was carried on the BALB/c background, an A_α^{g7} (i.e. A_α^d) gene was introduced at the same time. All B6xBALB/c F1s carrying both the TCR transgenes and the A_β^{g7} transgene developed arthritis, while none of the littermates carrying one or the other did (Table 1). The disease that developed in KRN mice carrying the A_β^{g7} transgene resembled in all respects (penetrance, time

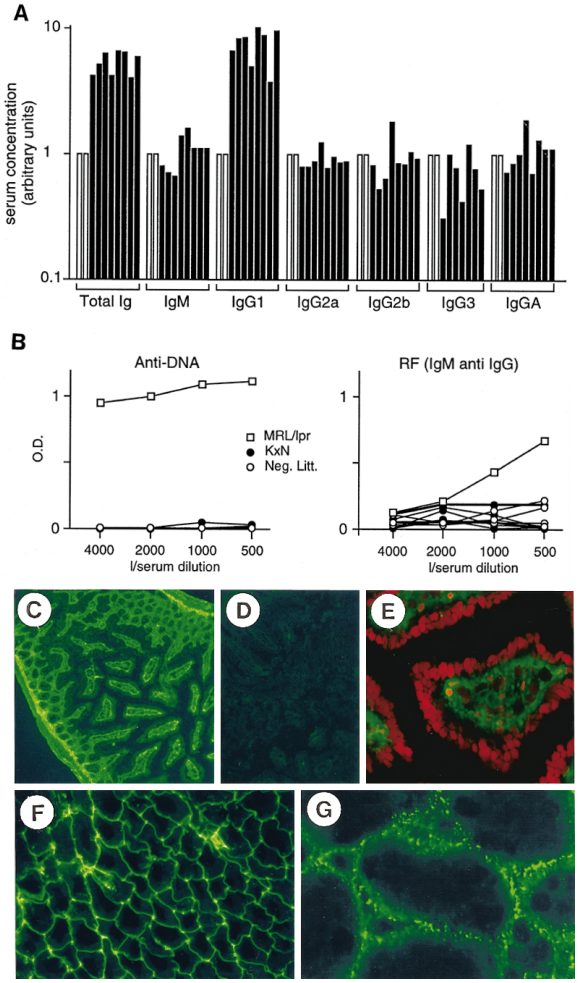


Figure 4. B Lymphocyte Alterations

(A) Levels of circulating Ig isotypes in 8- to 20-week-old transgenics (closed bars) normalized relative to negative littermates (open bars).
(B) Anti-DNA and -IgG autoantibodies in sera of transgenics, negative littermates, and a positive control MRL/lpr mouse.
(C–G) Ig deposits detected by immunofluorescence with anti-IgG/FITC on cryostat sections. In the gut (C–E) IgG deposits were observed in the submucosa and within the lamina propria of the villi, along the subepithelial and pericapillary basal membranes; in the muscle, deposits were in the connective areas of the endomysium and perimysium around the fibers (F). In the kidney, there was some IgG in the glomerulae, but most was found in the form of granular deposits along the peritubular basal membranes in the cortex and medulla (G). Cell nuclei in section (C) counterstained with propidium iodide (red) highlighting the epithelial cell border. (A), (B), and (D), low power 100× view. (C) and (E), 400× on a confocal microscope. All from transgenics except (B), which was a negative littermate.

course, histology) that in KRN mice harboring the entire complement of NOD genes.

It appears, then, that the NOD-derived MHC class II molecule A^{g7} is the element responsible for promoting arthritis in KRNxNOD mice.

Alloreactive Recognition of NOD APCs by the R28 TCR Is the Key to Disease Development

The critical and specific requirement for A^{g7} suggested that the transgene-encoded TCR might recognize an arthritogenic antigen uniquely presented by this molecule. During our initial attempts to test this notion, we discovered that the R28 TCR actually responds to NOD APCs alloreactively, i.e. in the absence of intentionally added antigen. Stimulators could be splenic APCs from NOD or B6.H-2^{g7} mice (Figure 5A) or a hybridoma between NOD splenocytes and the M12 B lymphoma line (not shown); responders could be either the original T hybridoma R28 (not shown) or lymph node T cells from KRN transgenics on the B6 background (Figure 5A). The alloresponse was always weaker than the reactivity to bovine RNase peptide offered by A^k-expressing APCs.

That this alloreactivity is the basis for arthritis development in KRNxNOD mice was strongly suggested by results from a series of crosses substituting other MHC alleles in place of the NOD-derived (Figure 5B). There was a perfect correlation between the appearance of arthritis in the F1 offspring and allorecognition of their APCs by the R28 TCR.

Table 1. Incidence of Disease Detected Correlated with Genetic Background

Genetic Background	Incidence of Arthritis
B6 × B6	0/150[a]
B6 × NOD	>300/300[b]
B6 × BALB/c	0/7[a]
B6 × DBA/2	0/17[a]
B6 × B6.H-2^{nod}	6/6[b]
B6 × BALB/c.tgA $^{nod}_{\beta}$	10/10[b]

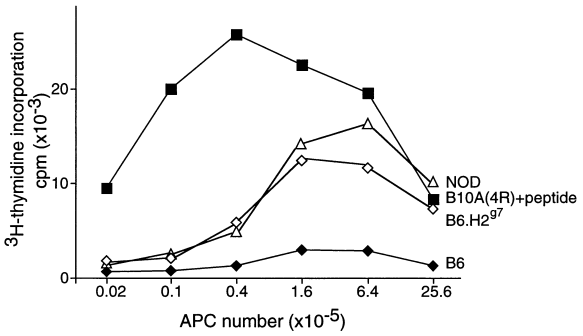
[a] No disease detected by inspection of the joints and by measure of ankle thickness. The absence of inflammation was verified in knee and ankle joints in some of these mice.
[b] Established by joint inspection and measure of ankle thickness, and histological confirmation in several mice. Arthritis was strictly dependent on the presence of the KRN transgene, and none of the negative littermates were affected. In all three instances, disease onset was between days 25 and 35.

T Cell Compartments in KRNxNOD Mice—Both Central and Peripheral Tolerization Are Evident, but Only Partial

Its reactivity to APCs from NOD mice means that the transgene-encoded TCR has autoreactive potential in KRNxNOD animals. Several examples of transgenic mouse lines carrying genes specifying autoreactive TCRs have been reported (Miller and Morahan, 1992). In most of them, T lymphocytes expressing the transgene-encoded receptors were tolerized by one or more means, e.g. clonal deletion, receptor or coreceptor down-modulation, anergy induction. We examined the T cells in KRNxNOD animals to see whether they were subject to analogous tolerization events.

Figure 6A compares numbers and CD4/CD8 profiles of thymus and lymph node cells in different-aged transgene-negative and -positive KRNxNOD mice. The youngest transgene-positive animals showed clear signs of clonal deletion in the thymus, evidenced by reduced cell numbers and aberrant CD4/8 profiles. The profiles progressively normalized thereafter, significant populations of mature single-positive cells being first observed at 3 weeks of age, although cell numbers remained slightly subnormal. Clonal deletion in the thymus

A



B

Genetic Background		Arthritis	Alloreactivity
B6 x B6	(b)	-	-
B6 x B10.A	(k)	-	-
B6 x B10.A (4R)	(k)	-	-
B6 x B10.BR	(k)	-	-
B6 x B10.M	(b)	-	-
B6 x B10.D2	(d)	-	-
B6 x B6.H2^{g7}	(g7)	+	+

Figure 5. Alloreactivity of the KRN Receptor and Its Relationship to Arthritis Development

(A) Lymph node cells from a KRN/B6 mouse were stimulated with graded numbers of splenocytes (for B10.BR, supplemented with 25 μg/ml RNase 41–61 peptide); proliferation was measured after 48 hr. (B) Correlation between the arthitogenic potential of the KRN transgene on different genetic backgrounds (the allele at the A locus of the MHC shown in parentheses) and alloreactive recognition by the KRN TCR, tested as in (A).

was reflected in the peripheral T cell compartments, as single-positive lymph node and spleen cells also did not appear in significant numbers until after 3 weeks, and the CD4$^+$ population never reached normal size (3.1-fold average reduction, n = 8; not shown).

Figure 6B examines TCR usage on peripheral CD4$^+$ cells of 7 week-old KRN transgenics carried on the B6 versus B6xNOD genetic background, or on the B6xNOD background bearing a homozygous null mutation of the endogenous TCR-α locus (Philpott et al., 1992). The goal was to determine whether muted levels of the transgene-encoded TCR might account for the ability of the potentially autoreactive T cells to escape from the thymus in older animals. In B6 mice, transgene-encoded V$_\beta$6$^+$ TCRs were expressed at high levels on most CD4$^+$ cells (upper box); however, there was a shoulder of reduced staining and this corresponded to cells displaying endogenously encoded V$_\beta$s (shown for V$_\beta$8 in the middle box). The lack of an appropriate reagent precluded quantitation of transgene-encoded V$_\alpha$4$^+$ receptors, but endogenously encoded V$_\alpha$2^{hi} TCRs were clearly detectable (lower box). In B6xNOD mice, CD4$^+$ cells displaying high levels of V$_\beta$6$^+$ TCRs were seen, but now the majority of cells showed intermediate or low levels (upper box). Again, the reduced staining corresponded to cells expressing endogenously encoded (e.g. V$_\beta$8$^+$) receptors (middle box) and, again, endogenously encoded V$_\alpha$2^{hi} receptors were easy to detect, predominantly on V$_\beta$6^{hi} cells (lower box). Introduction of the TCR-α null mutation onto the B6xNOD background had two effects. As expected, T cells no longer displayed V$_\alpha$2$^+$ TCRs (lower box)—the mutation permitted expression of transgene-encoded V$_\alpha$4 only, so all cells expressed this and only this V$_\alpha$. In addition, T cells displaying the highest levels of V$_\beta$6$^+$ TCRs were much rarer (upper and middle boxes). This implied that the CD4$^+$ T cells in KRNxNOD mice could express high levels of one or the other of the transgene-encoded chains, but usually not both. Hence, it seems quite plausible that thymocytes expressing the potentially autoreactive receptors escaped clonal deletion because incomplete allelic exclusion permitted rearrangement and expression of endogenous TCR-α and -β genes and thereby reduced levels of the transgene-encoded specificity.

Many of the peripheral cells in weeks-old KRNxNOD mice exhibited signs of prior activation (data not shown). A greater proportion expressed late activation markers (CD44^{hi}, CD62L^{lo}, CD45RB^{lo}) than in transgene-negative littermates (2–4 times), but there was only a small increase in the fraction displaying early activation markers (CD25^{hi}, CD69^{hi}).

Despite their increased expression of certain activation markers, the peripheral T cells from KRNxNOD mice appeared functionally compromised: although normally reactive to polyclonal stimulators like anti-CD3, they exhibited only meager clonotypic reactivity. As illustrated in Figure 7A, lymph node cells from B6 transgene-positive animals made a vigorous response when offered bovine RNase 41–61 by APCs expressing A^k. In striking contrast, cells from transgene-positive B6xNOD animals responded very poorly, although a weak reactivity was reproducibly observed (in all of 5 experiments) at the highest peptide concentrations (inset). RNase-specific cells were poorly responsive rather than poorly represented in KRNxNOD mice because the majority of hybridomas derived from lymph node cells after polyclonal stimulation with anti-CD3 produced IL-2 when challenged with the bovine RNase peptide in the presence of APCs expressing A^k (Figure 7B). Dose–response curves for hybridomas derived from transgene-positive B6 and B6xNOD animals overlapped. Hybridomas that responded to the bovine RNase peptide presented by A^k could also be stimulated alloreactively by NOD APCs (not shown).

Finally, although technically demanding, it was possible to examine the few T cells present in ankle synovial fluid. The most important observations were that: (1) there was a shift in the CD4/CD8 profile in comparison with that characteristic of lymph node, spleen, or blood lymphocytes, with a marked enrichment for CD4$^+$ cells (Figure 6C); (2) the CD4$^+$ population was enriched for cells displaying high levels of the transgene-encoded receptor (Figure 6D). (Although the latter observation might be taken as indicative of a clonotype-specific response in the joint, it is equally consistent with the absence of a response and consequent TCR down-regulation.) Too few synovial fluid T cells could be isolated to test their reactivity in vitro.

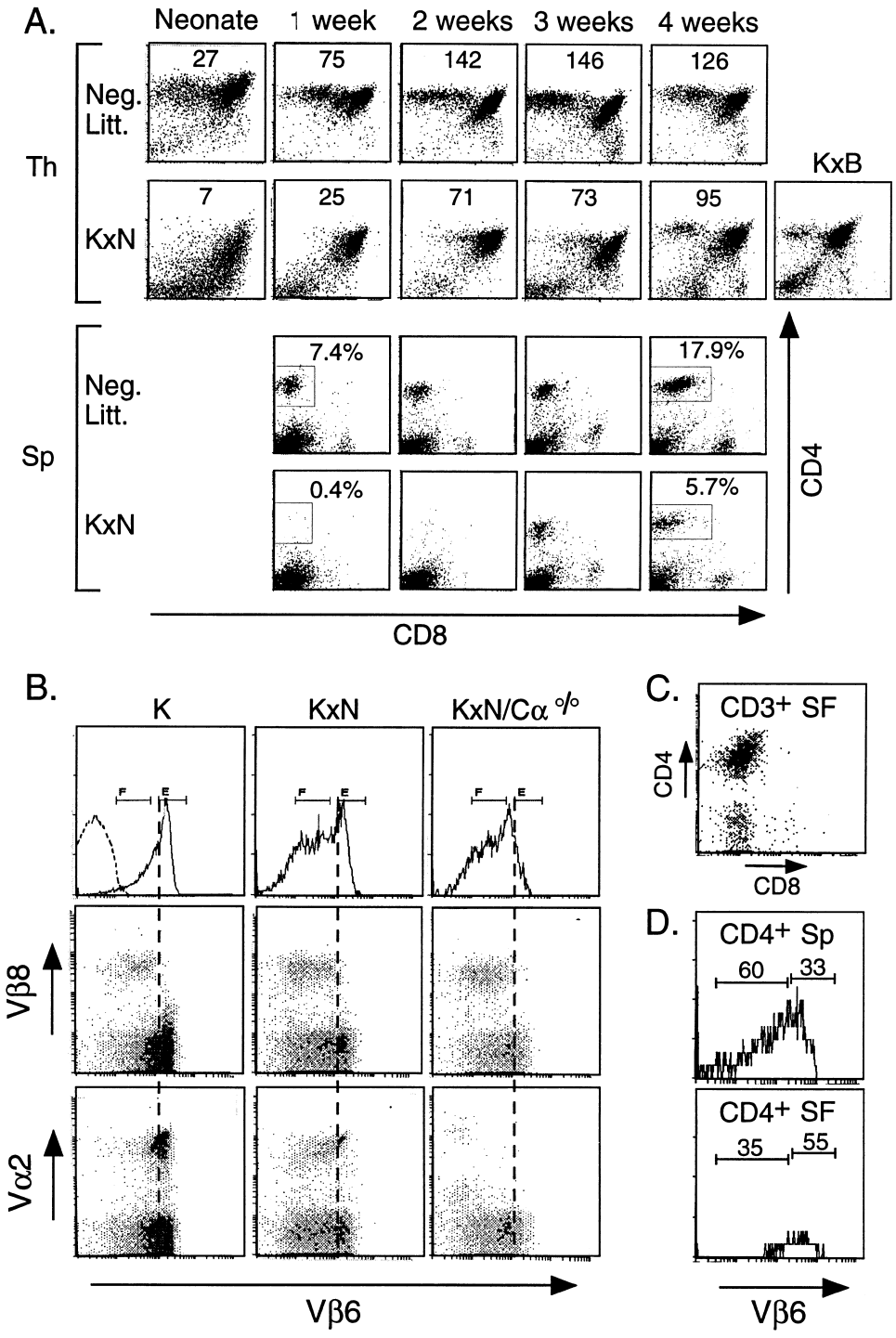


Figure 6. T Cell Compartments

(A) Thymocytes (Th) or splenocytes (Sp) from KxN transgenics and negative littermates or a KRNxB6 (KxB) mouse stained for CD4 and CD8. The values in the thymus panels represent the total cell numbers $\times 10^{-6}$, those in the spleen panels, the percentages of $CD4^+$ cells.

(B) Gated $CD4^+$ splenocytes stained for transgene-encoded Vβ6 (top panels; the [E] and [F] gates delineate high and intermediate Vβ6 levels). Coexpression of Vβ6 with nontransgenic Vβ8 or $V_\alpha 2$ is shown on the dot-plots aligned below, the vertical line helping to visualize $V_\beta 6^{hi}$ cells. Shown are a KRN/B6 mouse (K), a KRNxNOD (KxN), or a KRNxNOD carrying a homozygous null mutation at the TCR-α locus.

(C) CD4/CD8 profile of T cells in the ankle synovial fluid of an arthritic mouse. Electronically gated as $CD3^+MAC\text{-}1^-$.

(D) Vβ6 profile of gated $CD4^+$ cells in the spleen (Sp) or synovial fluid (SF).

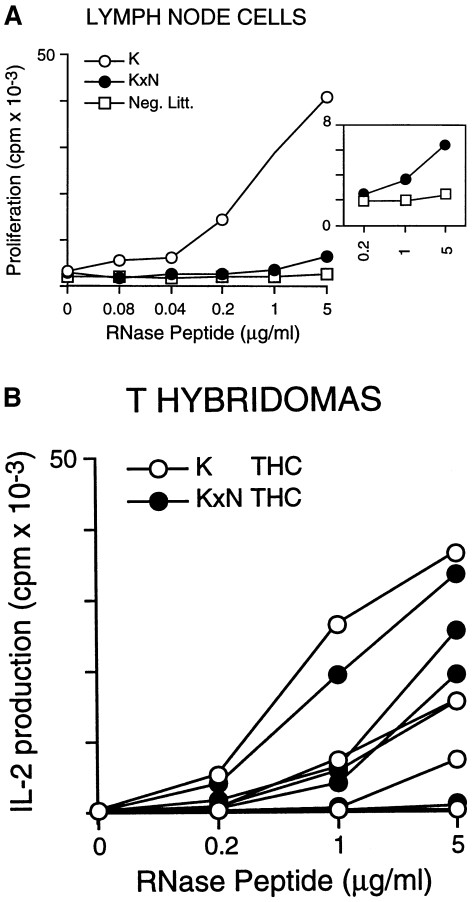


Figure 7. Clonotypic Reactivity of T Cells

(A) Lymph node cells from various mice (abbreviations as in Figure 6) were challenged with bovine RNase 41–61 peptide presented by B10.BR splenic APCs. The weak proliferation of KRNxNOD responders is highlighted in the inset, on an expanded scale.

(B) T cell hybridomas prepared from the indicated mice were incubated with bovine RNase 41–61 peptide and B10.BR splenic APCs. Stimulation was measured as IL-2 production, read-out as proliferation of CTLL.

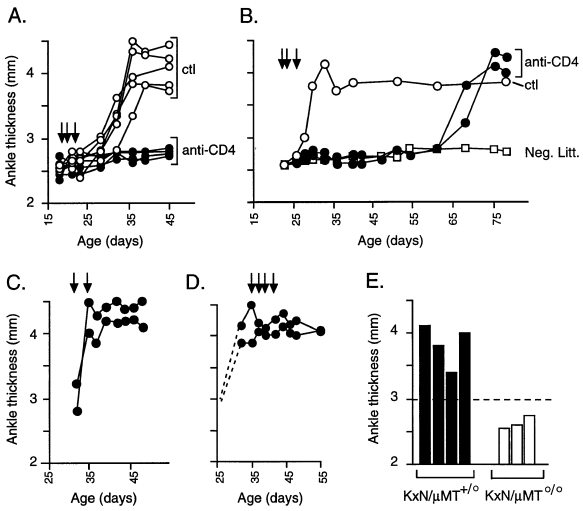


Figure 8. Requirement for CD4+ T and B Cells for Arthritis Onset

(A) Transgenics were treated by intraperitoneal injection of the anti-CD4 mAb YTS177 (Cobbold et al., 1990), titrated to block CD4+ cells for three to four weeks, or vehicle only on the indicated days (arrows). Disease progression was followed by measurement of ankle thickness.

(B) As in (A), except that mice were followed over a longer period.

(C and D) As in (A), except that treatment was initiated later.

(E) The μMT° mutation was introduced into KRNxNOD mice either in the phenotypically normal heterozygous state (closed bars) or the B cell–deficient homozygous state (open bars). Ankle thickness was measured at 40 to 50 days. That all μMT homozygotes were entirely free of disease was confirmed in five mice by histological analysis.

Taken together, the results on T cells from KRNxNOD mice indicate that the potentially autoreactive specificity is subject to two levels of tolerance induction: clonal deletion in the thymus and some form of anergy induction in the periphery. Clonal deletion was efficient only until about 3 weeks of age; shortly afterwards, mature T cells with muted levels of the autoreactive receptor escaped into the periphery and, nearly coincidently, the first signs of arthritis appeared.

The Role of Lymphocytes in Disease Development—CD4+ T and B Cells Are Required

The very nature of the KRNxNOD disease implies a critical role for T lymphocytes, i.e. arthritis only appeared when two elements were introduced onto the B6 background: the TCR genes originating from the R28 hybridoma and the A^{g7} genes. In addition, disease was first detectable just after significant numbers of mature T cells displaying transgene-encoded receptors emerged into the periphery. Yet, the paltry clonotype reactivity exhibited by lymph node T cells from KRNxNOD animals and the paucity of T cells in the arthritic lesion begged for an independent confirmation of the importance of T lymphocytes.

To this end, we treated KRNxNOD mice about one week before the usual time of disease onset with a nondepleting anti-CD4 mAb under conditions demonstrated (not shown) to completely coat CD4+ cells for 3 to 4 weeks. As indicated in Figure 8A, treatment according to this protocol led to a complete block of disease initiation. That the blockade was only temporary is depicted in Figure 8B: about 5 to 6 weeks after anti-CD4 treatment (and 4 to 5 weeks after disease routinely sets in) arthritis began; this corresponded to the reemergence of substantial numbers of cells stainable with the anti-CD4 mAb (not shown). Anti-CD4 had to be administered at least five days before disease onset to be effective; mice treated just at the time of (Figure 8C) or after (Figure 8D) initiation developed the usual arthritis.

Given the B lymphocyte abnormalities in both KRN xNOD mice and human RA patients, we evaluated the influence of B cells on the development of arthritis in our model. This was done by introducing the μMT° mutation (Kitamura et al., 1991) in homozygous form, preventing the surface display of Ig heavy chains, resulting in the absence of a mature B cell compartment. KRNxNOD mice devoid of B cells showed no signs of arthritis, evaluated either by joint measurements (Figure 8E) or histological analyses (not shown). Although B cells were

absent, T cells displaying the transgene-encoded receptor underwent clonal deletion in the thymus, and transgene-encoded receptor levels on peripheral T cells were reduced as in KRNxNOD animals with a normal complement of B cells (not shown).

Discussion

A New Model of Rheumatoid Arthritis

KRNxNOD mice represent a new model of RA in man as they spontaneously develop a highly reminiscent disease. Just how good a model is it?

Arthritis in KRNxNOD mice shares most of the major clinical, histological and immunological features of the human disorder. It is a chronic progressive disease that is first manifest by swelling of the joints, and evolves to a point where multiple joint deformities significantly impair mobility. Articular involvement is strikingly symmetrical and shows a marked distal preeminence, both characteristics of the human syndrome. Histological analysis reveals the classical images of massive leukocyte infiltration, synovitis, pannus formation, cartilage destruction, bone erosion followed by remodeling, and fibrosis. Immunological evaluation demonstrates B lymphocyte dysregulation, evidenced by increased B cell numbers and hypergammaglobulinemia. In addition, the critical dependence of the mouse disease on the MHC class II A^{g7} molecule recalls the long-studied influence of particular class II alleles on the disorder in man (Wordsworth, 1992).

Nonetheless, the arthritides of KRNxNOD animals and human subjects differ in several details. Clinically, there are a few discrepancies in the pattern of joint involvement: the mice show attack of the distal interphalangeal joints and, sporadically, inflammation of the spine, while humans generally do not. Histologically, there may be dissimilarities in the composition of the inflammatory infiltrate, as the large excess of myeloid cells over T lymphocytes and plasma cells in the inflamed synovial membrane of KRNxNOD mice appears more extreme than usually described for patient material. (Although patient histology, itself, is highly variable, making it difficult to assess the real magnitude of this dissimilarity.) Immunologically, the most striking discordance is in the profile of autoantibodies detected: RF, present at elevated concentrations in $\sim$70% of RA patients, is not observed in KRNxNOD animals; conversely, IgG deposits coat many of the organs in the mouse model but this has not been reported for patients. However, the significance of RF and other autoantibodies in arthritis pathology has been questioned (Feldmann et al., 1996b), and the murine/human differences could just reflect species-specific variations in B cell physiology—variant outcomes of massive B cell stimulation.

In evaluating the significance of these differences, one should bear in mind several considerations. Mice and men obviously show dissimilarities in physiology—perhaps most relevant here would be their divergent postures and modes of locomotion. Thus, it is not too surprising that a disease of the joints might differ in details. In addition, RA in humans is a highly variable syndrome, showing significant diversity in a number of parameters. The influence of genetics and environment on the panoply of abnormalities is not understood at present; so it is quite conceivable that the disease in genetically pure mice, housed in controlled environmental conditions, might not reproduce every aspect of the disorder in patients. A related point is that the mouse disease is more aggressive than the RA typically found in man, such that sites remaining intact in the latter (perhaps under the influence of drug treatment) are compromised in the former.

One needs also to situate the KRNxNOD strain in the context of other models of RA (for reviews, see Henderson et al., 1995). A variety of induced small animal models have been described, elicited by such agents as bacteria, viruses, adjuvants or cartilage components. On the other hand, few spontaneous models have been reported and most of these have not been explored in great depth. Mice of the MRL/lpr strain, the most extensively studied example to date, often show histological, though rarely clinical, signs of polyarthritis, and exhibit high concentrations of circulating RF and other autoantibodies (Andrews et al., 1978). However, this is primarily a lymphoproliferative disease, due to an alteration in the *fas* gene, and is probably more akin to systemic lupus erythrematosis than RA. DBA/1 mice can spontaneously develop polyarthritis (Nordling et al., 1992), but it is asymmetric, intermittent, and migratory, and shows little evidence of immune system involvement. Two strains of mice exhibiting diseases reminiscent of RA were recently engineered via transgenesis. In one (Iwakura et al., 1991), the HTLV-1 genome was introduced; the animals develop a chronic polyarthritis, but it is slow in onset and only partially penetrant (50% incidence at 1 year in females; even lower in males). In the other (Keffer et al., 1991), the transgene was a human TNF-α gene modified in the 3′-untranslated region; these animals show a chronic, destructive polyarthritis at an early age with 100% incidence but, curiously, the same disorder is exhibited by transgenics rendered devoid of an immune system (cited in Feldmann et al., 1996a).

Thus, it would appear that the KRNxNOD model represents a potentially important tool for studying RA—both for exploring the mechanism of disease development and for testing new therapeutic strategies. Its usefulness should be enhanced by several features: 100% disease incidence, an early and reproducible time of onset, a highly predictable disease course. In addition, its being a TCR transgenic system highlights and greatly simplifies the contribution made by lymphocytes and should thus permit new approaches to dissecting the role of the immune system in pathology.

The Mechanism of Arthritis Development in KRNxNOD Mice

The R28 T cell hybridoma responds alloreactively to APCs from NOD mice due to recognition of the MHC class II A^{g7} molecule; consequently, T cells from mice carrying rearranged TCR genes from R28 and A^{g7} genes from the NOD strain should exhibit systemic autoreactivity. That they do is readily apparent from an analysis of T lymphocyte compartments in KRNxNOD mice. The

thymus shows a clear deletion profile until 3 weeks of age; around that time, mature T cells appear in the thymus and periphery, although their numbers never quite reach normal levels. Peripheral T cells are characterized by a reduced display of the transgene-encoded TCR (coupled with frequent expression of endogenously encoded receptors) and by poor responsiveness. Nevertheless, their emergence coincides with the first clinical and histological signs of arthritis. Thus, one gets the impression of autoreactivity that is largely contained, but not totally eliminated, by multiple tiers of tolerance induction. Why deletion is so partial after 3 weeks is unknown and will probably remain so until we learn more about the exact ligand recognized—A^{g7} irrespective of peptide? plus multiple peptides? plus a single peptide? (We do know that the ligand is not the corresponding mouse RNase peptide presented by A^{g7}). Presumably, some alteration in the population of thymic stromal cells or in their expression of A^{g7} or its peptide complement occurs around this time, perhaps related to the vast hormonal changes taking place at weaning. That the partiality of clonal inactivation may be key is suggested by the KRNxDBA/2 cross, which introduced the $mls1^a$ superantigen: offspring exhibited clonal deletion more extreme than that found in KRNxNOD mice and no signs of arthritis were apparent (Table 1).

The low level of systemic autoreactivity persisting in KRNxNOD mice is manifest as an organ-specific disease. Although we do not yet know how this comes about, we do have in hand several pieces of information that provide important leads. The anti-CD4 experiments demonstrate a critical role for $CD4^+$ T cells and further reveal that they perform their required function(s) very early in disease course—anti-CD4 treatment just prior to or after disease onset provides no benefit. The studies on mice carrying the μMT° mutation, and thereby lacking a mature B lymphocyte compartment, show an important role for B cells. This could be either as antibody producers or as antigen presentors, or could reflect some more indirect function. Lastly, joint histology and cytokine profiles argue for the involvement of cells of the myeloid lineage, perhaps as producers of inflammatory factors such as TNF-α and IL-6.

Considered in their ensemble, these leads suggest several possible scenarios for the development of arthritis in the KRNxNOD model. First, the key to joint specificity could lie in the abnormal activation of B lymphocytes: essentially all B cells constitutively display A^{g7} and should thus be recognized by T cells expressing the transgene-encoded TCR and be delivered "help" that is aberrant in its universality and route of delivery, i.e. independent of the Ig receptor. The outcome could be high level synthesis, either systemically or locally, of an unusual combination of lymphokines and other mediators, which might promote synovitis and recruitment of leukocytes into the joint. Second, aberrant activation of a cell population residing in the normal synovium could be the basis of joint specificity. Polyclonal activation of both T and B lymphocytes should take place in KRNxNOD mice due to mutual stimulation via the TCR–MHC class II molecule interaction, resulting in differentiation, lymphokine secretion, and altered homing properties. The lymphokines produced, e.g. IFN-γ, TNF-α, IL-4, would be capable of inducing class II molecule expression on a variety of cell types that usually display none or little, including synoviocytes. Aberrant activation of these cells could then result from recognition of their A^{g7} molecules by the transgene-encoded TCR, and this activation might set off a sequence of events leading to arthritis. It is highly suggestive that MHC class II molecules are lymphokine-inducible on macrophages and synovial fibroblasts and that their engagement leads to the production of a variety of mediators, including TNF-α, IL-1β, nitric oxide, IL-6, and IL-8 (Alvaro-Gracia et al., 1989; Trede et al., 1991; Mourad et al., 1992; Mehindate et al., 1994). Third, specificity could be imposed by the physiology of the joint: it is poorly vascularized and subject to mechanical stress, factors that might combine to create an environment of low oxidative state. Redox balance is known to modulate the synthesis or activity of transcription factors expressed by T cells (Ivanov et al., 1993) and is suspected to underlie the control that bcl-2 exerts on apoptosis (Hockenbery et al., 1993). Thus, it is possible that different programs unfold when T cells in the joint, versus those in the lymphoid organs and other tissues, are stimulated—in the former case, biased towards proliferation and cytokine production; in the latter, towards anergy or death. Alternatively, or in addition, the poor vacularization and mechanical stress of the joint could result in ineffectual clearance of immune complexes issuing from polyclonal B cell activation and, by consequence, stimulation of the complement cascade and induction of inflammatory cytokines.

Whatever the mechanism turns out to be, one aspect should be highlighted—systemic autoreactivity can provoke an organ-specific disease. Wide distribution of the inciting antigen/A^{g7} complex is indicated by the deletion of immature T cells in the thymus and by the ability of splenocytes to stimulate R28 T cells in vitro. That the distribution does not just reflect the binding of circulating antigen by A^{g7} displayed on APCs is suggested by the finding that a B hybridoma derived from NOD splenocytes and cultured for months also stimulates R28 cells in vitro.

Implications for the Mechanism(s) of Disease Development in Other Models and Patients

One is led to question whether the disease in other RA models or in RA patients might not arise from a similar systemic, rather than joint-specific, provocation.

Certainly there are some models where the mechanism of disease is likely to involve primarily systemic stimulation. For example, the arthritis in animals infected by mycoplasma arthritides could unfold in a very similar fashion to that in KRNxNOD mice. Upon infection, this microbe produces a superantigen, mycoplasma arthritis mitogen, which can bridge MHC molecules and TCRs or directly trigger cells through their MHC molecules, thereby inciting polyclonal T and B cell activation and the production of inflammatory cytokines (TNF-α, IL-1, IL-6) by monocytes. This superantigen has already been directly implicated in arthritis exacerbation (Cole and Sawitzke, 1995). Another model to consider in this context is the transgenic mouse line carrying a modified human TNF-α gene (Keffer et al., 1991). These animals

express TNF-α in a deregulated fashion throughout the body and develop an arthritis apparently quite similar to that in KRNxNOD mice, except that it appears to be independent of lymphocytes (cited in Feldmann et al., 1996a). We suggest that the diseases in the two models might depend on a similar mechanism of pathogenesis, but that the TNF-α transgenic model just initiates further downstream, after any inciting systemic autoreaction. The HTLV-1 transgenic model (Iwakura et al., 1991) might also fit somewhere along this pathway, as high levels of IL-1, probably induced by the virally encoded Tax protein, have been reported in these animals.

There are also suggestions that systemic reactivity might play a role in human rheumatoid arthritis. Quite analogous to the observations on KRNxNOD mice are recent findings that a significant fraction of synovial T cells from several patients recognize antigens expressed by autologous APCs (David-Ameline et al., 1996; Schmidt et al., 1996), in particular B cell antigens encoded by Epstein-Barr virus (Scotet et al., 1996). In addition, TCR V_β usage in certain patients suggests stimulation by superantigens (reviewed in Cole and Sawitzke, 1995). In such cases, polyclonal activation and expansion of T cells could be coupled to a massive reciprocal activation of B cells and, as discussed above, this could be a trigger for arthritis development. In this context, it might be worth reconsidering the basis for RA association with particular MHC alleles: some alleles, due to peculiarities of expression or protein structure, could more readily provoke stimulation of autologous T cells.

Thus, it is quite possible that the mechanism of disease in KRNxNOD mice does accurately reflect that in RA patients. On the other hand, we know of no compelling evidence for the involvement of joint-specific antigens in the human disorder. The challenge now is to fill in the missing mechanistic details of the mouse model in order to elaborate the complete pathway from systemic provocation to joint-specific disease.

Experimental Procedures

Mice

The $A\beta^{g7}$ transgenic line on the BALB/c background was a gift from D. Wherrett and H. McDevitt. The B6.H2^{g7} congenic line was provided by H. Kikutani. The μMT° and TCRa° knockout lines (Kitamura et al., 1991; Philpott et al., 1992), gifts from K. Rajewsky and M. Owen, were back-crossed in parallel onto the B6 and NOD/Lt backgrounds. The B6 back-cross animals were crossed to KRN, and the resulting $Tg^+KO^{+/o}$ progeny further crossed to $KO^{+/o}$ mice on the NOD background to generate $Tg^+KO^{o/o}$ and corresponding control animals. Ankle thickness was measured with a J15 Blet micrometer.

DNA Constructs and Transgenesis

The sequences of the variable regions of the α and β chains of the R28 hybridoma were determined as previously described (Candeias et al., 1991). From this information, rearranged $V_\alpha4J\alpha27$ and Vβ6Jβ1.5 genomic DNA fragments were amplified and cloned into TCR expression cassettes (Kouskoff et al., 1995), yielding plasmids paKRN and pbKRN. Large fragments were excised and injected into fertilized B6xSJL F2 eggs. Screening of the transgenic founders was performed on Southern blots with an 800 bp XmaI–NotI VJα probe from paKRN or with a 1.6 kb EcoRI probe from the germline Jβ2 region. A single founder in which TCRα and -β transgenes were cointegrated was identified.

Antibodies and Flow Cytometry

Preparation of thymus, lymph node, and spleen cell suspensions, their staining for flow cytometry, and the mAbs used were as described (Cosgrove et al., 1991; Wang et al., 1996). Synovial fluid cell suspensions were made after several ankle punctures; the viscous fluid leaking out (3–20 μl) was diluted in PBS/EDTA and centrifuged.

Determination of circulating Ig levels was performed by ELISA (Cosgrove et al., 1991). Assays for autoantibodies included immunofluorescence on cryostat sections of mouse liver and kidney (1/50 serum dilutions, goat anti-mouse-IgG/FITC second step), ELISA with dsDNA or mouse IgG as target antigens (with goat-anti-mouse-IgG$^+$M/phosphatase or anti-mouse-IgM/peroxydase as second steps).

Histology

Joints were fixed in 4% paraformaldehyde, decalcified for 8 hr in 6% nitric acid followed by 8 hr in 4% paraformaldehyde (treatment repeated twice), and embedded in paraffin; 2 μm sections were stained with hematoxylin/eosin/safran. Immunostaining of cryostat sections was performed as described (Cosgrove et al., 1991), with mAbs M1/70-FITC (Pharmingen), BM8 or MOMA-1 (Kraal and Janse, 1986) or KT3 (developed with anti-rat-IgG/Texas red), or anti-mouse-IgG/FITC. For skeletal preparations, limbs or fragments were fixed overnight in 1% glacial acetic acid, 95% ethanol, stained for cartilage with fresh Alcian blue solution (80 ml 95% EtOH, 20 ml glacial acetic acid, 15 mg Alcian blue) for 48 hr. After dehydratation in 95% ethanol for a day, soft tissues were dissolved in 2% KOH for a few hours at 37°C. Bones were stained in 1% KOH, 75 mg/ml Alizarin red overnight, destained in 20% glycerol, 1% KOH for 7–15 days. Remaining loose tissues were dissected away and the preparations stored into 50% glycerol, 50% EtOH.

T Cell Stimulation and Hybridomas

Lymph node T cells were cultured at 3×10^5/well in 96-well plates in RPMI-1640 medium supplemented with 1 mM glutamine, 50 μM 2-ME and 10% FCS, for 36–48 hr at 37°C. Proliferation was detected by ^{3}H-thymidine incorporation in the last 12 hr of culture. The KRN receptor was stimulated by varying doses of peptide 41–61 of bovine pancreatic RNase (Peccoud et al., 1990) or with B6xNOD or B6xB6.H2^{g7} splenocytes.

The M12xNOD hybrid cell line was obtained by PEG fusion of LPS-blasts (24 hr culture) with the M12 B lymphoma line; hybrids were selected in HAT medium and by flow cytometry after staining with the anti-$A\beta^{g7}$ mAb 10.2.16 (C. Ebel, unpublished).

To derive T cell hybridomas from the transgenic mice, spleen or lymph node cells were stimulated with anti-CD3 for 24 hr in vitro, fused to BW5147α-β-, and the hybrids selected in HAT medium. These hybridomas were tested for IL-2 production after 24 hr stimulation in the presence of 3×10^5 B10.BR splenocytes and RNase peptide 41–61, or of graded numbers of NOD splenocytes. IL-2 production was detected by incorporation of ^{3}H-thymidine by the CTLL indicator line.

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