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Breakdown of CTL Tolerance to Self HLA-B*2705 Induced by Exposure to Chlamydia trachomatis

Igor Popov,*† Charles S. Dela Cruz,‡ Brian H. Barber,§ Basil Chiu,* and Robert D. Inman2*†

There is a strong association between seronegative arthritis and HLA B27, but it is still unresolved whether the contribution of B27 to disease pathogenesis is solely as a restriction element for an arthritogenic peptide, or whether B27 itself serves as an autoantigen. This study uses transgenic rats to address the question as to whether exposure to an arthritogenic pathogen can alter tolerance to B27. Unlike their nontransgenic counterparts, B27-transgenic rats are tolerant of B27 immunization using either B27+ splenocytes or plasmid DNA and do not develop anti-B27 CTL. However, if splenocytes from such immunized animals are exposed to Chlamydia in vitro, CTL are generated that lyse B27+ targets. No killing was seen with targets transfected with control B7, B14, B40, or B44. This phenomenon was not observed with immunization by nontransgenic splenocytes, or HLA-A2 DNA alone. Using targets expressing mutated B27, we show that the epitope for autoreactive CTL recognition of B27 involves the Lys70 amino acid residue in the α1 domain of the MHC class I molecule. The generation of CTL with specificity for B27 under these conditions demonstrates that tolerance to B27 can be subverted by Chlamydia. This indicates a dynamic interrelationship between the pathogen and B27, which may have important implications for B27-related spondyloarthropathies triggered by intracellular bacteria. The Journal of Immunology, 2002, 169: 4033–4038.

Although the presence of the HLA class I allele B27 predisposes humans to the spondyloarthropathies (SpA) (1), it is unresolved whether the contribution of B27 to disease pathogenesis is solely as a restriction element for an arthritogenic peptide, or whether B27 itself may serve as an autoantigen. Besides genetic predisposition, a role for environmental factors appears very likely (2, 3). This is highlighted by the triggering of reactive arthritis, a subset of SpA, after infection with certain intracellular pathogens, such as Chlamydia, Yersinia, Shigella, Salmonella, and Campylobacter (4, 5). One central aspect of SpA pathogenesis that remains to be clarified is the mechanism by which the B27 genetic background can specifically influence the immune response to such arthritogenic pathogens (6).

It is known that Ag derived from intracellular bacteria may lead to activation of CD8+ lymphocytes via class I-mediated pathways (7), thereby invoking CD8+ CTL in a central role in resistance to these bacteria (8). CTL recognizing bacteria-derived peptides presented by self B27 are relevant in host defense (9, 10). Such a B27-dependent event might figure critically in the clearance of an arthritogenic agent. Alternatively, the CTL response might be directed to a self peptide, thereby initiating an autoimmune process (11). Presentation by HLA class I molecules of closely related peptides from B27 and bacteria could lead to T cell responses to self B27 or inactivation of antibacterial T lymphocytes (12). The peptide specificity of B27 also seems to figure critically in the spontaneous arthritis seen in certain lines of B27 transgenic (Tg) rats (13). Although the role of CTL in recognizing MHC class I-restricted Chlamydia peptides has been studied in both clinical and experimental systems (14–17), these studies have generally not sought to address the interrelationship with concurrent B27-related immune responses. Kuon et al. (18) recently identified B27-restricted peptides from the Chlamydia proteome that were demonstrated specificity for CTL derived from B27-Tg mice. Addressing this issue in the current study, we used a Tg rat model involving both Chlamydia and B27 to study the influence that host genetic background may have on the process of generating anti-Chlamydia and anti-B27 CTL.

Materials and Methods

Animals

The Tg rat line 21-4L, bearing six copies each of the B*2705 and human β2-microglobulin (hβ2m) on the inbred Lewis (LEW) background (RT11), was a kind gift of J. Taurog (University of Texas Southwestern Medical Center, Dallas, TX) (19). Non-Tg inbred LEW rats, 2–3 mo old, were purchased (Harlan Sprague Dawley, Indianapolis, IN). Rats were maintained in microisolators, and screened by FACS of their PBMC to confirm appropriate MHC expression.

Antibodies

For flow cytometry, mAbs were derived from different hybridomas (American Type Culture Collection (ATCC), Manassas, VA): HB24 (anti-H-2Dα), HB95 (W6/32, reacting to all HLA-I in association with hβ2m), HB119 (ME-1, recognizing B27 as well as B7 and Bw22), and HB82 (BB7.2, anti-HLA A2). The mAbs purified from hybridoma W3/25 (MCA55G, specific to rat CD4) and MRC OX-8 (MCA48G, rat CD8 specific) (Serotec, Raleigh, NC) were used for the in vitro blocking of the appropriate T subsets.

Isolation of genes

Full-length A2 and B27 cDNAs were generated by standard RT-PCR using mRNA isolated from splenocytes of Tg mice. To amplify the A2 and B27 genes, PCR was conducted, as previously described (20).

Abbreviations used in this paper: SpA, spondyloarthropathy; Ct, Chlamydia trachomatis; EB, elementary body; hβ2m, human β2-microglobulin; LEW, Lewis; Tg, transgenic.

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3 Abbreviations used in this paper: SpA, spondyloarthropathy; Ct, Chlamydia trachomatis; EB, elementary body; hβ2m, human β2-microglobulin; LEW, Lewis; Tg, transgenic.
Plasmid DNA immunogens

A2 and B27 PCR products were cloned into the expression vector pcDNA3 (Invitrogen, San Diego, CA). Two eukaryotic expression vectors were constructed: pcDNA3-A2 and pcDNA3-B27 (18). The vector constructs, encoding class I HLA, were characterized by restriction enzyme analysis and confirmed by sequencing with T7 polymerase (Pharmacia, Piscataway, NJ). The completed plasmid DNA was ampliﬁed in the JM109 Escherichia coli and puriﬁed using EndoFree Megaprep Kit (Qiagen, Chatsworth, CA).

In vitro cell surface expression of A2 and B27 proteins was conﬁrmed by COS-7 cell transfections, according to previously described methods (21).

Immunization of rats

Groups of four B27-Tg rats were immunized with each of the following immunogens: B27-Tg splenocytes, non-Tg splenocytes, B27 DNA, A2 DNA, B27.71TNT (the mutation of Lys 70 into Gln mutation), or mutant B27.AKA69 tor DNA alone, or non-Tg LEW splenocytes (data not shown). The rats were immunized at days 0 and 21, and subsequently boosted at day 42. Spleens were harvested for CTL activity measurements 3-4 wk postboost.

Mixed lymphocyte culture

Spleen cells from harvested immunized rats were cocultured in vitro for 5 days with stimulator splenocytes from naive B27-Tg rats at 5:1 responder/stimulator ratio. In some experiments, stimulators (1-2 x 10^3) were treated with 10^6 Chlamydia trachomatis (Ct; LGV type II, strain 434) elementary bodies (EB) (Microbix Biosystems, Toronto, Ontario, Canada) washed once and added into 96-well round-bottom microtiter plates (Falcon 3077; BD Biosciences, Lincoln Park, NJ) (10^5 cells/well in 100 µl medium). A total of 100 µl/well of serial dilutions of in vitro restimulated effectors was also added. Spontaneous ^51Cr release was measured in wells with targets and nonimmune spleen cells. Plates were centrifuged at 100 x g for 5 min, incubated for 6 h at 37°C under 5% CO2, and averaged 14% at any E:T ratio. SD was always <5% specific lysis. The spontaneous release of 10^6 Cr of targets incubated alone did not differ from wells in which targets were incubated with nonimmune spleen cells.

Targets

Synovial ﬁbroblast lines were generated in non-Tg and B27-Tg rats using induced synovitis, as we described (22). The murine L cell line J26 (ATCC) transfected with hβ2 was previously cloned. Its clone J26.6 was transfected with genomic DNA encoding B14, B27, B40, B44, or mutant B27.C67Y (mutating Cys67 into Tyr), as we also described (23, 24). Mouse L cells, LTK-, transfected with hβ2, m and B7, mutant B27.K70Q (the Lys70 into Gin mutation), or mutant B27.AKA69-71TNT (the mutation of Ala69Lys/Ala71 into ThrAsnThr), were a kind gift from J. Taurog (25). B14, B40, and B44 were selected as controls having no known relationship to SpA. The cell lines are checked periodically by FACS to ensure stable expression of the transgenes. In some experiments, targets were incubated with Ct (10^3 EB/ml) for 2-3 h at 37°C in humidified 95% air/5% CO2 and assaying, as described above.

Blocking of T lymphocyte subsets in vitro

Anti-CD4 or anti-CD8 mAbs at a concentration of 1 µg/ml were added to restimulated splenocytes at 10^7/ml, and maintained in complete medium for 1 h at +4°C.

Results

Prior exposure to self B27 reduces the threshold for generating anti-Chlamydia CTL

Irradiated naive B27° Tg LEW spleen cells were injected i.p. into syngeneic rats. The animals were boosted as described, and the primed splenocytes were restimulated in vitro with the Tg cells used for the in vivo immunization. In contrast to our previous studies using non-Tg rats, B27° Tg animals were tolerant of immunization and restimulation with the self B27° cells, as reﬂected in the absence of any CTL killing of B27° targets. The T cell tolerance was also observed when a plasmid DNA construct encoding full-length B27 was used, instead of the splenocytes, for in vivo immunization via an i.m. route (Fig. 1, A and C). The ﬁgure is representative of three to five experiments. We had previously demonstrated that both the plasmid DNA and cellular strategies were effective in generating CTL against B27 in non-Tg LEW rats (26).

To address the inﬂuence that self B27-related immunity may have on the process of generating anti-Chlamydia CTL, splenocytes from either syngeneic cell-immunized or B27 DNA-immunized rats underwent a 5-day restimulation in vitro with Chlamydia-treated B27-Tg spleen cells. We observed that splenocytes from Tg animals immunized with either syngeneic cells or the plasmid DNA construct were now shown to generate an anti-Chlamydia CTL response. There was CTL killing of Chlamydia-sensitized non-Tg LEW ﬁbroblast targets, but this was observed only when the effectors had been restimulated in vitro with Chlamydia-treated B27-Tg cells (Fig. 2). There was no killing of non-Chlamydia-sensitized LEW targets with these effectors. Naive spleen cells could not be shown to generate a primary anti-Chlamydia immune response by in vitro stimulation of Chlamydia-primed Tg or non-Tg stimulators. In contrast, in vitro generation of anti-Chlamydia CTL could be induced if animals had ﬁrst been primed in vivo with B27-Tg cells or B27 DNA, but not with non-Tg splenocytes, A2 DNA, or vector DNA alone (data not shown).

Induction of autoreactive anti-B27 CTL follows in vitro exposure to Chlamydia

We next addressed the speciﬁcity of CTL obtained from B27-Tg rats primed in vivo with either syngeneic cells or B27 DNA and thereafter restimulated in vitro with Chlamydia-treated B27-Tg splenocytes. As expected, killing was observed, with recognition of both Chlamydia-sensitized B27° rat ﬁbroblasts (Fig. 1A) and murine L cells (Fig. 1C). When cytotoxicity against nonsensitized B27-Tg rat and B27° murine cell targets was examined, we observed killing of all of the B27° targets. Killing was comparable, whether (Fig. 1, B and D) or not (Fig. 1, A and C) the targets had ﬁrst been sensitized with Chlamydia. No cytotoxicity was found on non-Tg LEW synovial ﬁbroblasts and murine L cells transfected with third party B44. Furthermore, no such killing was seen with splenocytes from control animals immunized with HLA-A2, vector DNA alone, or non-Tg LEW splenocytes (data not shown). These observations point toward the existence of autoreactive anti-B27 CTL.

Inhibition of CTL specific to Chlamydia and self HLA-B27 by anti-CD8 mAb

We evaluated whether CD4° or CD8° cells were responsible for the lysis of Chlamydia° and B27° targets. Splenocytes from B27-Tg animals challenged in vivo with B27 DNA, then stimulated in vitro with Chlamydia-treated B27° cells were incubated
with anti-CD4 or anti-CD8 mAb. Lysis of non-Tg fibroblasts sensitized with *Chlamydia* was decreased by 61% with the anti-CD8 treatment of effector cells. For the corresponding experiment using B27-Tg rats injected either with syngeneic splenocytes or with B27 DNA, and restimulated in vitro with B27-Tg spleen cells either treated with Ct EB (Ct⁺) or untreated (Ct⁻).

**Defining the specificity of the autoreactive CTL**

To determine whether the autoreactive anti-B27 CTL recognition was specific for self B27, we used a panel of L cell targets transfected with hβ₂m and HLA-B allele controls other than B44. We observed no killing by the autoreactive CTL of targets expressing B7, B14, or B40 (Fig. 3, lanes 5–7). The effect of CTL recognition of mutated amino acid residues in the hypervariable region of the α₃ domain of B27 was then examined. Killing by the autoreactive CTL was still observed for targets expressing B27.C67Y, to a degree comparable as native B27 (Fig. 3, lanes 1–2). Thus, the autoreactive CTL did not discriminate the Cys→Tyr mutation at position 67. However, these CTL did not recognize targets...
expressing B27.AKA69–71TNT mutant molecules (Fig. 3, lane 4). Indeed, the single residue mutant B27.K70Q was not recognized, indicating that mutation of B27 Lys3Gln at position 70 alone was sufficient to abrogate the autoreactive B27-specific CTL recognition (Fig. 3, lane 3).

**Discussion**

We have recently shown that prior expansion of an in vivo immune response against B27 in LEW rats reduced the threshold for generating a primary anti-Chlamydia CTL response (26). To address the role of reactivity to self-B27 vs nonself, in this study we examined this phenomenon in B27-Tg animals.

Rats Tg for B27 and hβ2m have proved useful for investigating the basis of B27-associated SpA. Tg rat lines expressing high levels of B*2705/hβ2m develop a spontaneous disease with striking resemblance to human SpA, and this disease is critically dependent on B27-bound peptides (18). Tg rats from lines that express lower copy numbers of B27 develop no spontaneous disease. In the present study, we have used the 21-4L, one such low-copy line on LEW background.

As was seen in the xenogeneic B27 response observed with our non-Tg animals (24), we have also observed in this study that prior exposure to self B27 results in a reduced threshold for generating a primary in vitro CTL response to Chlamydia. Unexpectedly, this precondition of in vitro exposure to Chlamydia led to induction of the autoreactive CTL with specificity for B27, in the animals otherwise tolerant to self B27, by a single exposure in vitro to Chlamydia following the in vivo priming with self B27. These data implicate a dynamic interrelationship between immune recognition of Chlamydia and breakdown of tolerance to self B27. This phenomenon might be explained by cross-reactive recognition of self and microbial peptides. CTL with either of these two specificities may play a pathogenic role in B27-associated SpA. The arthritogenic peptide model (27) for the pathogenesis of SpA postulates that the B27-binding motif sets the stage for presentation of peptides to potentially pathogenic CTL. These peptides might be of endogenous origin or exogenous origin or both, and cross-recognition of such peptides might be favored by B27-restricted T cells.

| Table I. Blockinga of CTLb specific to Ct and self B27 by anti-CD8 mAb |
|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | B27-Tg LEW Cells | B27 DNA          |
| Target Cells     | +Anti-CD8 (%)    | +Anti-CD4 (%)    | +Anti-CD8 (%)    | +Anti-CD4 (%)    |
| LEW fibroblasts  | 40               | −9c              | 61               | −2c              |
| B27-Tg LEW fibroblasts | 49               | 12               | 39               | 5                |
| B27-transfected murine L cells | 44               | −7c              | 43               | −12c             |
| Ct+              | 55               | 15               | 37               | −12c             |
| Ct+              | 43               | −11c             | 50               | −3c              |

a Percent inhibition = ([1 − (% lysis with specific mAb by immune cells − % lysis with specific mAb by naive cells)/% lysis with control Ab by immune cells − % lysis with control Ab by naive cells]) × 100%.

b E:T ratio was 100:1.

c −, Refers to increase in CTL killing.
This notion of potentially cross-reactive peptides is supported by the observation that B27-restricted CTL with specificity for both self and bacterial peptides can be found in synovial fluid of SpA patients (28). The notion of such cross-reactivity has been proposed to be a central factor in the pathogenesis of B27-related SpA (29).

The interrelationships of infection and autoimmune complex, and may invoke a variety of pathways in addition to molecular mimicry (12). It has been argued that mimicry may arise as a secondary phenomenon caused by alteration of host antigenic determinants through tissue injury and the creation of neoepitopes. This process of tissue injury uncovering cryptic epitopes does not seem pertinent to our present observation of in vitro stimulation. Activation of otherwise anergic autoreactive T cells can result from up-regulation of costimulatory molecules on APCs, being a necessary precondition for T cell activation in conjunction with cognate binding of the MHC-peptide complex by TCRs. It is evident that autoreactive T cells do survive thymic deletion strategies to some extent, as must be the case for our B27-Tg rats. Peripheral tolerance mechanisms (30) then become the means of keeping such cells in check and preventing an ongoing autoimmune assault on host tissues.

Our studies on the mutant B27 targets implicate Lys at position 70 as being critical for an epitope recognized by the autoreactive CTL. In the studies of Lopez de Castro and colleagues (31, 32), the residue Lys70 plays a key role in the specificity of CTL. It was of interest that in our study the Cys70Tyr mutation did not affect autoreactive CTL recognition of B27. A Cys70Ser mutation of B*2705 has been felt to still predispose Tg animals to arthritis in vivo (34). Studies in progress in our laboratory are addressing whether the epitope of xenoreactive anti-B27 CTL differs from that of autoreactive anti-B27 CTL.

Activation of quiescent autoreactive T cells might implicate a sequence homology between the respective peptides from Chlamydia and B27, but this may not necessarily be the precondition for cross-reacting T cell responses. Studies by Wucherpfennig and Strominger (35) demonstrate that T cell clones may show significant cross-reacting TCRs. It is of interest that the residue Lys70 plays a key role in the specificity of CTL. Tertiary structure, hydrophobicity, and electrostatic charges are all potential contributors to interactions with cross-reacting TCRs. It is of interest that the residue Lys70 does not point upward, in a way that would reflect direct contact with the TCR, but forms a salt bridge with residue Asp74, which in turn figures critically in peptide binding (28, 29). The elution studies by Jardetzky et al. (36) indicate that a significant population of peptides normally resident in the B27 peptide-binding cleft is derived from B27 itself. It remains a distinct possibility that the B27-mediated sequelae of Chlamydia infection such as reactive arthritis are fundamentally autoimmune processes. Antibiotic therapy does not alter the natural course of this arthritis (37), nor has recent experience with anti-TNF therapies unmasked any occult or subclinical infectious arthritis (38). Thus, if the persistent inflammation is maintained by a sustained source of microbial peptides, the source of such exogenous peptides is obscure. Our results provide experimental evidence of a break in tolerance to B27 occasioned by exposure to Chlamydia. This may provide a mechanistic bridge between what begins as a septic event, but which evolves into an autoimmune process. It also raises the possibility that peptide therapy may be a feasible approach to treating this condition in the future.

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