Brief Definitive Report

CYTOTOXIC T LYMPHOCYTES AGAINST DISEASE-ASSOCIATED DETERMINANT(S) IN ANKYLOSING SPONDYLITIS

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The serological crossreactivity between certain enteric bacteria and HLA-B27 (1, 2) and/or an associated structure (3, 4) on the cells of patients with HLA-$B27^+$ ankylosing spondylitis ($B27^+$,AS^+) has been documented by some groups in three different populations (5–7), but its relevance to the pathogenesis of AS remains obscure. An extension of these studies by our group has shown that HLA-$B27^+$ lymphocytes from normal individuals ($B27^+$,AS^-), which are not lysed by crossreactive antisera (i.e., antisera that specifically lyse $B27^+$,AS^+ cells [3, 4]), can be rendered susceptible to lysis after incubation in the culture filtrate (containing modifying factor) of crossreactive bacteria (8, 9). One possible consequence of the modification by bacterial antigens of an HLA-B27-associated structure is the production, by genetically susceptible individuals, of CTL that would kill targets bearing HLA-B27 in association with certain bacterial antigens. The destruction of target tissues (e.g., synovium and other connective tissues) by cytotoxic mechanisms might lead to an inflammatory reaction in the sacroiliac and spinal joints, which is a characteristic lesion in AS. In support of this possibility, we report that CTL, raised by stimulating the PBMC of an $B27^+$,AS^- individual with the PBMC of an HLA-A-, -B-, -C-identical sibling suffering from AS, will specifically lyse $B27^+$,AS^+ PBMC but not PBMC from HLA-$B27^+$ or $B27^-$,AS^- normal individuals or from HLA-$B27^-$ AS patients ($B27^-$,AS^+). Moreover, these disease-specific CTL will lyse $B27^+$,AS^- PBMC that have been modified in vitro with culture filtrate from crossreactive bacteria. CTL of similar specificity can also be raised by immunizing $B27^+$,AS^- cells with autologous cells modified by crossreactive antigens in vitro.

Materials and Methods

Patients. All HLA-$B27^+$ and HLA-$B27^-$ patients were suffering from classical ankylosing spondylitis as defined by the New York criteria (10). HLA-$B27^+$ and HLA-$B27^-$ healthy controls were members of staff and blood donors with no history of joint disease.

Bacterial Cultures. Klebsiella K43 BTS 1, a transformant of *E. coli* C600 that has

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acquired by transformation a gene encoding modifying factor (*E. coli* C600-pBB1; J. S. Sullivan, J. K. Prendergast, L. I. Upfold, and A. F. Geczy, manuscript submitted for publication) and an *E. coli* from the bowel flora of an HLA-B27$^+$ AS patient, were grown in nutrient broth No. 2 or Luria broth (Oxoid, Basingstoke, England) with vigorous aeration for 16–20 h at 37°C. The culture filtrates were harvested by centrifugation at 5,000 *g* for 20 min, concentrated 20-fold with polyacrylate gel (11), and filtered through a 0.2 μm pore size membrane (Millipore, Bedford, MA).

Production of CTL to HLA-B27-associated Determinants. PBMC were obtained from groups of HLA-B27$^+$ ($n = 12$), and HLA-B27$^-$ ($n = 4$) patients with ankylosing spondylitis, as well as from HLA-B27$^+$ ($n = 14$) and HLA-B27$^-$ ($n = 10$) normal controls. The cells were separated from heparinized blood by centrifugation in Lymphoprep (Nyegaard, Oslo, Norway) for 25 min at 580 *g*, and washed in RPMI medium containing 10% human AB serum (3). CTL were raised by incubating 10^7 mononuclear (fresh or frozen/thawed) cells (responder cells) with 8–10 × 10^6 in vitro–modified or unmodified (e.g., B27$^+$,AS$^+$ PBMC) mitomycin-treated autologous or allogeneic mononuclear (stimulator) cells in 20 ml of RPMI medium and 20% human AB serum. The responder and stimulator cells were incubated in 50-ml tissue culture flasks (Corning Glass Works, Corning, NY) at 37°C for 6–7 d in an atmosphere of 5% CO_2 in air. Mononuclear cells cultured for 6–7 d in the same medium without any stimulant were used as targets.

For the production of CTL to in vitro–modified HLA-B27, mononuclear cells (10^7 B27$^+$,AS$^-$ in 1.9 ml of RPMI medium containing 20% human AB serum) were incubated with 20-fold concentrated culture filtrate (0.1 ml) for 12–16 h at 37°C. The culture filtrates were obtained from one of the crossreactive organisms, including *Klebsiella* K43 BTS 1, an *E. coli* from the bowel flora of a B27$^+$,AS$^+$ patient (Fig. 1*A*), as well as an *E. coli* transformant that had acquired a plasmid bearing a gene(s) encoding modifying factor (*E. coli* C600-pBB1). After modification with culture filtrate, the PBMC were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO; 40 μg per 8–10 × 10^6 cells) for 30 min at 37°C, washed twice with RPMI medium and 20% human AB serum, and used as stimulator cells. When required as targets, they were incubated for 5–6 d in medium alone and then modified for 12–16 h before use.

For the production of CTL to the putative disease-associated determinant, B27$^+$,AS$^-$ PBMC (8–10 × 10^6) were incubated with mitomycin-treated B27$^+$,AS$^+$ PBMC (8–10 × 10^6) from an HLA-A-, -B-, and -C-identical AS-affected sibling. After a 6–7-d incubation, the CTL assay was run for 5–6 h at 37°C in round-bottomed 96-well plates using 10^4 ^{51}Cr-labeled target cells (0.1 ml) and effector cells (0.1 ml) at various E/T ratios (50, 10, and 5:1). The amount of ^{51}Cr released into the supernatant was measured in a gamma counter. Percent specific release was calculated as 100 × [(experimental release) − (spontaneous release)]/[(total release) − (spontaneous release)]. Spontaneous release was 15–30% of the detergent control (total release).

CTL Competition Assay. Cold or unlabeled targets were treated the same as hot or ^{51}Cr-labeled targets cells, except that they were not labeled with ^{51}Cr. Cold targets (0.1 ml) were added to individual wells containing effector cells (0.05 ml) before the addition of ^{51}Cr-labeled target cells (0.1 ml). The cold target cells were added at various cold/hot cell ratios from 5:1 to 50:1.

Blocking of CTL Activity with Antibodies. Alloantisera (anti-HLA-B27 and -B44), mAb (HLA-ABC M1, monomorphic anti–class I, 1 mg/ml; lot 418202, A.M.D., Artarmon, New South Wales, Australia) or normal human AB serum were added to individual wells of microtiter plates together with 10^4 target cells. After a 45-min incubation at 37°C, effector cells were added and the cultures were incubated for an additional 5 h before harvesting.

Results and Discussion

Previous studies from our group (4, 8) have documented the specific modification of B27$^+$,AS$^-$ lymphocytes by a factor (modifying factor) in the culture filtrate of certain crossreactive bacteria. We wished to determine whether CTL

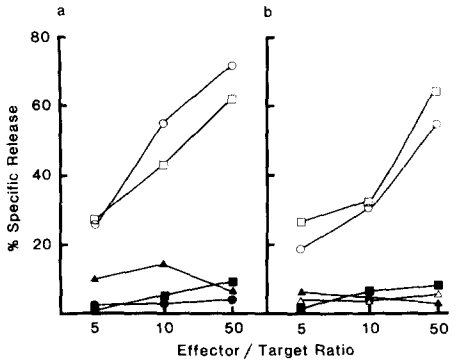

FIGURE 1. CTL raised to: (*a*) autologous in vitro–modified $B27^+,AS^-$ PBMC, and (*b*) a disease-associated determinant developed by sensitizing the PBMC of a $B27^+,AS^-$ normal individual (A2, -; B27, Bw62; Cw1, Cw3) with PBMC of an HLA-A-, -B-, and -C-identical sibling suffering from AS. The target cells used in these studies were: $B27^+,AS^-$ PBMC modified with crossreactive culture filtrate (○), $B27^+,AS^+$ PBMC (□), $B27^+,AS^-$ PBMC (unmodified) (■), $B27^-,AS^+$ PBMC (▲), $B27^-,AS^-$ PBMC modified with crossreactive culture filtrate (●), $B27^-,AS^-$ PBMC (unmodified) (△).

could be raised to crossreactive determinants in the context of HLA-B27. The culture filtrates with this modifying factor activity were obtained not only from the previously characterised isolate *Klebsiella* K43 BTS 1, but also from a crossreactive *E. coli* isolated from the bowel flora of an AS patient. This *E. coli* might be of particular relevance to the pathogenesis of AS, since fecal isolates of *E. coli* with crossreactive determinants have so far been recovered from all 52 patients with AS, but from only one of 50 $B27^+,AS^-$ controls (12). The culture filtrate of a transformant of *E. coli* C600 was also used to modify $B27^+,AS^-$ PBMC. This isolate of *E. coli* has acquired by transformation a gene(s) encoding modifying factor (*E. coli* C600pBB1; J. S. Sullivan, J. K. Prendergast, L. I. Upfold, and A. F. Geczy, manuscript submitted for publication). As culture filtrates of all crossreactive bacteria have similar modifying factor activity (4, 13) we considered it reasonable to use these filtrates interchangeably.

Fig. 1*A* shows that CTL, raised to $B27^+,AS^-$ cells that had been modified in vitro with culture filtrate of an *E. coli* from the bowel flora of a $B27^+,AS^+$ patient, will kill not only $B27^+,AS^-$ cells treated with culture filtrate, but also $B27^+$ cells from AS patients. However, these CTL will not lyse the cells of $B27^-$ AS patients or of unmodified $B27^+$ normal controls. The cells of $B27^-$ normal controls that have been modified with culture filtrate are also not lysed, suggesting that the crossreactive determinants are seen only in association with HLA-B27.

As an extension to the studies on in vitro–modified $B27^+,AS^-$ PBMC, we attempted to raise CTL to the putative disease-associated determinant by stimulating cells from an HLA-$B27^+$ normal individual with cells from an HLA-identical sibling suffering from AS (the diagnosis of AS was based on the New York criteria [10]). The results in Fig. 1*B* show that CTL to this disease-associated structure will lyse $B27^+,AS^+$ PBMC well as in vitro–modified $B27^+,AS^-$ PBMC. By contrast, the PBMC of $B27^-$,AS patients and of $B27^+$ and $B27^-$ normal controls are not killed, indicating that these CTL recognize a hypothetical disease-associated structure only in the context of HLA-B27.

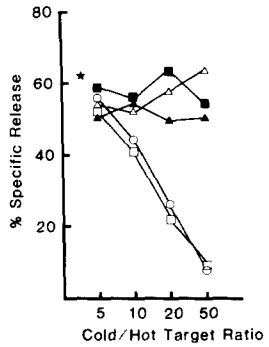

FIGURE 2. Cold target inhibition studies of CTL directed against the disease-associated determinant on $B27^+,AS^+$ PBMC. The E/T ratio was 25:1 and the unblocked activity is indicated by a star (★). Cold targets (0.1 ml) were added to wells containing effector cells (0.05 ml) before the addition of ^{51}Cr-labeled target cells (0.1 ml). The cold target cells were added at various cold/hot cell ratios from 5:1 to 50:1. The following cold target cells were tested: $B27^+,AS^-$ PBMC modified with crossreactive culture filtrate (○), $B27^+,AS^+$ PBMC (□), $B27^+,AS^-$ PBMC (unmodified) (■), $B27^-,AS^+$ PBMC (▲), $B27^-,AS^-$ PBMC (unmodified) (△). The CTL activity was assayed as described in Materials and Methods.

CTL to the B27-associated determinant on $B27^+,AS^+$ PBMC have so far been raised in two families: in one family, the patient and the normal sibling were, HLA-A2, -; B27, Bw62; Cw1, Cw3 (see Fig. 1*B*) and in the other family: HLA-A2, Aw33; B27, Bw71; Cw1, Cw2 (results not shown).

These CTL, like the crossreactive antibacterial sera (3, 4), recognize a similar or an identical determinant on $B27^+,AS^+$ cells and on in vitro–modified $B27^+,AS^-$ cells. It is likely, therefore, that CTL will prove to be useful typing reagents for the detection of B27-associated determinants on the cells of patients and their family members. To confirm the specificity of the CTL raised to $B27^+,AS^+$ PBMC, cold target inhibition studies were performed, and these results are illustrated in Fig. 2. It is clear that only $B27^+,AS^+$ and modified $B27^+,AS^-$ PBMC are able to block CTL activity, while no blocking is seen in the presence of cold targets from the three other categories of PBMC (i.e., $B27^-,AS^+$, $B27^+,AS^-$, and $B27^-,AS^-$).

To demonstrate further the requirement for HLA-B27 in the B27-associated complex recognized by CTL, alloantisera and a monomorphic anti–class I serum were used to block CTL activity. The data in Table I show that the CTL activity of effector cells against the B27-associated determinant can be blocked when modified $B27^+,AS^-$ target cells are exposed to an monomorphic anti-class I serum or to an antiserum to HLA-B27. In contrast, anti-B44, which is also expressed on the target cell, failed to block CTL activity.

The observations reported here indicate that the PBMC of patients suffering from ankylosing spondylitis carry an HLA-B27-associated determinant that can be specifically recognized by CTL. These CTL, which have so far been tested on 12 $B27^+$, 4 $B27^-$ AS patients, and on 10 $B27^+$ and 10 $B27^-$ normal controls, are specific for modifying factor or the disease-associated determinant in association with HLA-B27.

The specificity of the CTL documented in this report raises a number of interesting possibilities with respect to the initiation of the disease in genetically

TABLE I

Ability of Anti-HLA-B27 Serum to Block CTL Activity against a B27-associated Determinant

Serum	Percent specific release		Inhibition (%)‡	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Human AB (1:5)*	65	58	—	—
Anti-HLA-B27 (1:5)	14	23	78	60
Anti-HLA-B44 (1:5)	73	57	0	0
Anti-class I (monomorphic) (1:50)	18	16	72	72

Effector cells were produced by stimulating $B27^+$, AS^- PBMC with in vitro-modified autologous PBMC; target cells were modified $B27^+$, AS^- PBMC (for details see Materials and Methods).

* Final concentration of serum or antibody in microtiter wells during CTL assay.

‡ Percent inhibition = 100 × (1− specific release in presence of antiserum)/(specific release in presence of control serum).

susceptible individuals. It is tempting to suggest that CTL specific for modified HLA-B27 might destroy target tissue bearing HLA-B27, in association with certain factors derived from crossreactive bacteria, and thereby induce an inflammatory reaction. IF CTL or similar cytotoxic mechanisms play a role in the pathogenesis of AS, then perhaps some connective tissues are better targets than others possibly because they present to the effector cells a more recognizable or compatible structure.

To our knowledge, this is the first demonstration of CTL to HLA-B27 modified by bacterial products, and we suggest that these CTL may represent a plausible effector mechanism in the pathogenesis of AS.

Summary

Cytotoxic T lymphocytes, induced by stimulating the PBMC of an $HLA\text{-}B27^+$ normal individual ($B27^+$,AS^-) with the PBMC of an HLA-identical sibling suffering from ankylosing spondylitis (AS) ($B27^+$,AS^+), specifically lyse $B27^+$,AS^+ PBMC but not PBMC from $HLA\text{-}27^+$ or $B27^-$,AS^- normal controls, or from $HLA\text{-}B27^-$ AS patients ($B27^-$,AS^+). CTL of similar specificity can also be raised by immunizing in vitro $B27^+$,AS^- cells with autologous cells modified by cross-reactive bacterial antigens. These results suggest that CTL can recognize certain bacterial antigens in association with HLA-B27 and that this interaction may lead to an inflammatory episode during the initial stages of the disease.

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