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THE USE OF FUSION PROTEINS TO STUDY HLA-B27-SPECIFIC ALLORECOGNITION

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Abstract—Potential antigenic regions of the various external domains of the HLA-B27 antigen were expressed as fusion proteins in bacterial hosts and analyzed for their ability to induce humoral and cellular responses. Monoclonal antibodies directed against the proteins recognized monomorphic determinants of denatured HLA-antigens, but not B27-antigens expressed by intact lymphocytes. T-cell proliferation and IL-2 secretion were induced with a fusion protein representing regions of the first and second domains around amino acid residue 114. None of the fusion proteins stimulated cytotoxic T-lymphocytes (CTL) in an HLA-specific manner, although several included those amino acid sequences thought to be important for CTL recognition.

INTRODUCTION

While B-lymphocytes directly bind native antigens with their immunoglobulin receptors, T-cells only recognize processed antigenic peptides which are presented to their receptors in association with self-MHC molecules (Zinkernagel and Doherty, 1979). The MHC molecules present peptides to T-cells with a distinct class related bias: class I molecules predominantly activate cytotoxic T-lymphocytes (CTL); whereas class II molecules primarily stimulate helper T-cells. Recent studies have emphasized that antigen presenting cells need to intracellularly process intact antigens into peptides which are bound by restricted sites of MHC molecules, forming a complex (self + X) (Allen, 1987). These complexes are transported to the cell surface where they are accessible to T-cell receptor recognition. Since a very high percentage of T-cells apparently have receptors specific for allogeneic MHC molecules (Lindahl and Wilson, 1977) the question arises whether alloantigens, as typical integral membrane proteins, are also intracellularly processed and seen as peptides in association with self-MHC molecules (Kourilsky and Claverie, 1986, Bjorkman *et al.*, 1987; Clayberger *et al.*, 1987) or whether conformation allows the display of an allogeneic stretch of residues sufficient to be recognized by T-cells as a self + X complex.

To investigate this question we studied responses by T-cells and humoral antibodies to the well known human alloantigen HLA-B27. For this purpose cDNA fragments of the B27 gene, encoding various segments of the first and second domains, were cloned into expression vectors and recovered as fusion proteins. The resultant fusion proteins included stretches of residues which, following processing, should provide important allogeneic determinants. They should also display limited conformational determinants, although these would be clearly distinct from those of the native molecules. These fusion proteins were used to study induction of monoclonal antibodies in mice *in vivo* and induction of human helper and cytotoxic T-cell subsets *in vitro*. The response of T-helper cells was measured by IL-2 secretion and CTL reactivity by lysis of allogeneic target cells. T-helper cells could be stimulated to secrete IL-2 with a fusion protein containing the combined first and second domains, while CTL were non-specifically activated by some fraction of the bacterial lysate. Monoclonal antibodies induced against the various fusion proteins precipitated HLA-class I antigens from cellular lysates, as shown in Western blot and isoelectric focussing, but were not specific for HLA-B27.

MATERIALS AND METHODS

Cloning of HLA-B27 cDNA fragments into expression vectors

B27 cDNA fragments (Szöts *et al.*, 1986) (Fig. 1) were cloned into the expression vectors pEx1 (Stanley and Luzio, 1984) and pEx10 (Seedorf *et al.*, 1987) providing transcripts fused with β galactosidase in the case of pEx1 and MS2 polymerase in the case of pEx10. Translation of fusion protein D3 will terminate at the end of $\alpha 3$ due to a stop codon: therefore D3 will only express a peptide corresponding to amino acid positions 180–274. The complete first domain could not be obtained since the cDNA clone used for these experiments started at amino acid 27

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and the restriction site at position 37 was chosen to eliminate the poly-T-tail resulting from the transcription of mRNA to cDNA. Correct fit of each B27-cDNA fragment into the reading frame of the expression vector was controlled by DNA sequencing with the method of Sanger *et al.* (1977).

Expression and purification of fusion proteins

Expression of fusion proteins was induced with a heat shock of 42°C [to inactivate the thermosensitive repressor of the *E. coli* strains pop (for pEx1) and 537 (for pEx10)]. Overnight cultures, grown at 30°C, were diluted four-fold with fresh medium which was prewarmed (45°C) to obtain a fast temperature shift. After shaking for 1–4 hr (42°C) the cultures were sedimented, the pellet resuspended in 1/10 vol glucose buffer (50 m*M* glucose, 10 m*M* EDTA, 25 m*M* Tris–HCl, pH 8.0) and sonicated for 2 min. After centrifugation for 20 min at 4000 *g* the pellet was resuspended in 1 *M* urea, sonicated and centrifuged again for 20 min at 4000 *g*. The resulting pellet was again resuspended in 7 *M* urea and sonicated for 2 min. Following centrifugation the supernatant was dialyzed against H_2O for 2–3 days, lyophilized and the protein weight determined. Fusion proteins were resuspended in phosphate buffered saline (PBS) and sonicated for 5 min to obtain a fine suspension.

Due to their insolubility, the fusion proteins were not easily purified by gel chromatography without prior treatment with strong detergents. For use in cellular assays they needed to be detergent-free, thus urea was used and subsequently removed by dialysis. The enriched fusion proteins, however, were contaminated by a fraction of urea soluble bacterial proteins.

All fusion proteins could be produced in high amounts compared to total bacterial protein, except for the unfused MS2 polymerase. The failure to induce high amounts of MS2 polymerase remains unexplained, although it may have been due to degradation by proteases.

Generation of monoclonal antibodies against fusion proteins

Six-week-old F1 mice (C57Bl/6 × BALB/c) were immunized twice i.p. at weekly intervals with a mixture of 50 μg each of the fusion proteins MS2-D1/2, -D1, -D2 and -D3, mixed with 10^9 *B. pertussis*. After 7 days they were boosted i.v. with the same amount of antigen alone and 3 days later the spleen cells were fused with the myeloma line X63 Ag8.653 according to the protocol of Hämmerling (1977). Supernatants were tested for binding to MS 2-fusion proteins as follows: round-bottom PVC-plates (Falcon, Becton Dickinson, Oxnard, CA) were coated (4°C, overnight) with 50–100 μl of MS 2-fusion protein, dissolved in 7 *M* urea and then diluted to a concn of 5–10 μg/ml PBS. After blocking with PBS containing 3% fetal calf serum (FCS) (37°C, 2 hr) and incubation with culture supernatants (4°C, overnight) a peroxidase-coupled rabbit anti-mouse-Ig antibody (P 161, Dianova, Hamburg) was added for 1–2 hr (37°C); *O*-phenylendiamine (Sigma, St Louis, MO) (1 mg/ml) dissolved in 0.1 *M* citrate buffer, pH 8, containing 0.01% H_2O_2 was used as a substrate for the peroxidase. Peroxidase activity was determined according to the substrate absorption at 450 nm.

Precipitation of HLA-antigens from cell lysates

Monoclonal antibodies were covalently crosslinked to rabbit anti-mouse-Ig-coupled Protein A-Sepharose according to the protocol of Gersten and Marchalonis (1978). Cellular lysates were prepared as described by Vasilov *et al.* (1983). HLA-antigens from cell lysates were incubated with the antibody-coupled Sepharose for at least 2 hr at 4°C. Before eluting the antigens with 50 m*M* desoxycholate (pH 11.2) the Sepharose was washed 3 × with NET (150 m*M* NaCl, 10 m*M* Tris–HCl pH 7.6, 1 m*M* EDTA, 0.5% NP 40). The eluate was then titrated to pH 7.4 with 1/10 volume of 0.5 *M* KH_2PO_4, mixed with SDS-sample buffer and applied on a 12.5% SDS–PAGE.

The Western blots were made according to Towbin *et al.* (1979). The blot was developed with the HLA-heavy chain specific monoclonal antibody HC 10 (Stam *et al.*, 1986; Neefjes, 1986), anti-mouse-immunoglobulin-coupled horse radish peroxidase (Dianova, Hamburg) and using sodium dianisidine and nitroprusside as substrate.

Isoelectric focussing

1.5×10^6 cells were labelled with 25 μCi ^{35}S-methionine. HLA-antigens were precipitated as described above but including a 4 hr preabsorption with rabbit anti-mouse-Ig-Protein A-Sepharose. After immunoprecipitation with the specific antibodies, the Sepharose complex was washed twice with each of the following buffers (A, B, C): A: 0.05 *M* Tris–HCl, pH 8.2, 1 m*M* EDTA, 0.5 *M* NaCl, 0.5% taurocholate; B: 0.05 *M* Tris–HCl, pH 8.2, 0.15 *M* NaCl, 0.5% taurocholate, 0.1% SDS; C: 0.05 *M* Tris–HCl, pH 8.2, 0.15 *M* NaCl, 0.5% taurocholate. The samples were treated with neuraminidase (Type VIII, Sigma, St Louis, MO) in reaction buffer (50 m*M* NaAc, pH 5.0, 1 m*M* $CaCl_2$) for 4 hr at 37°C. The antigen was eluted from the Sepharose by adding IEF-sample buffer and applied on an IEF-gel as described (Neefjes, 1986).

Preparation of antigen presenting cells (APC)

All cell cultures were maintained in RPMI 1640 in a 5% CO_2-humidified air atmosphere. Fresh peripheral blood of HLA-typed donors was defibrinated using sterile glass beads, serum was collected and the fibrin clots were incubated (37°C, overnight) in RPMI medium containing 15% autologous serum. Monocytes that had migrated from the fibrin clots were isolated by ficoll gradient centrifugation (Schendel and Wank, 1981). The interface cells were

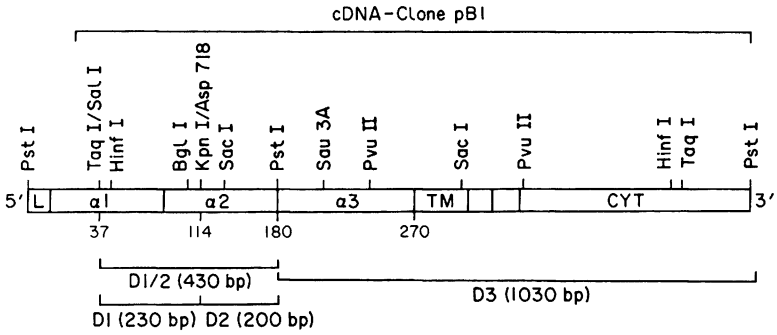

Fig. 1. Restriction map of B27-cDNA showing the cDNA-fragments used for the construction of the fusion proteins D1/2, D1, D2 and D3.

washed three times with RPMI prior to incubation with fusion proteins. Monocytes or Epstein-Barr-Virus-transformed B-lymphoblastoid cell lines (LCL) were incubated with 500–1000 μg of fusion protein per 10^6 cells in RPMI containing 10% autologous serum (for monocytes) or 10% FCS (for LCL) at 37°C for at least 6 hr or overnight. Before being used as antigen presenting cells the remaining fusion protein was removed by a ficoll gradient. LCL were irradiated with 5000 rad, monocytes with 2000 rad.

In vitro *stimulation of IL 2-secreting cells*

1×10^6 peripheral blood lymphocytes (PBL) were incubated with 2×10^5 allogeneic stimulator cells or fusion protein pulsed APC in 1 ml RPMI containing 10% pooled human serum. During primary stimulation, supernatants were collected after 18, 24, 48, 72 and 96 hr. For restimulation cells were harvested after 10 days, washed and incubated at a concn of 10^6/ml with the same number of irradiated PBL as stimulator cells. Supernatants were collected after 5, 14, 18, 24 and 36 hr. IL-2 content of the supernatants were measured by ^{3}H-thymidine incorporation of an IL-2 dependent cell line as described previously (Schendel and Wank, 1981). 2×10^4 IL-2 dependent cells were incubated in 50 μl RPMI 1640/10% pooled human serum plus 50 μl of test supernatant in round bottom microtiter plates. After 3 days incubation 2 μCi ^{3}H-thymidine were added per well and cultures were incubated for another 8 hr. Cells were then harvested and incorporated radioactivity was measured.

In vitro *generation of CTL*

5×10^6 PBL cells were incubated in an upright 50 ml culture flask with 5×10^6 allogeneic PBL (2000 rad) or 1×10^6 fusion protein-pulsed autologous APC (LCL or monocytes) in 7 ml RPMI 1640/10% pooled human serum. After 6 days cells were harvested and cytotoxicity was tested in a 4 hr chromium release assay on ^{51}Cr-labelled LCL or 3–4 d phytohemagglutinin (PHA) stimulated blasts. 10^3/100 μl ^{51}Cr-labelled target cells were incubated with 100 μl effector cells of varying dilutions in RPMI/10% FCS for 4 hr at 37°C. Spontaneous chromium release was determined by incubating labelled target cells in culture medium only and total release by directly measuring the labelled cells. Specific lysis (%) was calculated from the average of triplicates according to the following formula (Schendel *et al.*, 1979): (experiment ^{51}Cr release–spontaneous release): (max. ^{51}Cr release–spontaneous release) × 100.

RESULTS

Generation of fusion proteins

Our goal to obtain single, complete domains of the HLA-B27 chain was partially limited by the lack of appropriate restriction sites for cloning into the vector pEx1. As the restriction map of B27-cDNA demonstrates (Fig. 1), the second and the third domains could be easily separated, whereas no suitable restriction site was available between the first and second domains. Thus fusion protein D1/2 consisted of about half of the first domain and the complete second domain, whereas D1 is composed of half of the first and 1/3 of the second domain and D2 contained 2/3 of the second domain. The third domain (D3) was fully expressed and since the cDNA clone had a stop codon at the end of the third extracellular domain, the expression of the cytoplasmic and intracellular regions was avoided. The genes for MS2-polymerase or β galactosidase were fused to the 5'-end of the cDNA-segments.

Antibody responses to fusion proteins

The monoclonal antibodies generated against the various fusion proteins that were selected for further characterization are listed in Table 1. Only those were selected which showed differential binding to the various fusion proteins and did not react with the MS2-polymerase control fraction, with the exception of MS39, which reacted with all fusion proteins and MS2-polymerase. Antibody MS52 preferentially bound to fusion protein D3, whereas the others recognized D1/2, D1 and D3. One antibody recognized only D1 or D1/2, namely HC10 (Stam *et al.*,

Table 1. Binding of the monoclonal antibodies to the MS2-polymerase fusion proteins

	D1/2	D1	D2	D3	MS2
HC 10	+ + + +*	+ + + +	–	–	–
W6/32	–	–	–	–	–
MS 6	+ + + +	+ + + +	–	+ + + +	–
MS 10	+ + +	+ + +	–	+ + + +	–
MS 17	+ + +	+ + +	–	+ + + +	–
MS 32	+ + + +	+ + + +	–	+ + + +	–
MS 39	+ + + +	+ + + +	+ + +	+ + + +	+ + + +
MS 52	+ +	+ +	–	+ + + +	–

*The binding intensity was tested in ELISA by the absorption of the colored substrate at 450 nm. + + + + $E_{450} > 2.0$; + + + $E_{450} > 1.5$; + + $E_{450} > 1.0$; – E_{450} 0.0–0.3.

1986), a reagent that was generated against papain digested, sodium dodecyl sulfate denatured heavy chains of HLA-B. The binding of MS6, 10, 17 and 32 to both D1 and D3 suggested they share antigenic epitopes, although they have no similarities in amino acid sequence.

The monoclonal antibodies, W6/32 and HC10 were used to precipitate HLA-antigen from cellular lysates. Isolates of the mouse mastocytoma line P815 (Van Pel *et al.*, 1985) transfected with HLA-B27 (Kuon *et al.*, personal communication) as well as the untransfected control were tested in a Western blot (Fig. 2). The anti-fusion protein antibodies did not recognize SDS denatured antigens (data not shown), so they could not be used for development of the blots. When, however, the blots were developed with the HLA-heavy chain specific antibody HC10 (Stam *et al.*, 1986), bands corresponding to class I molecules were found in all precipitates of the transfected line, but not with the non-transfected cell. The same pattern appeared after precipitation of HLA-antigens from the LCL LG2 (HLA-A2, -B27, -Cw1) (unpublished observations).

Further analysis of the antigens precipitated from the HLA-homozygous LCL LG2 (A2, B27, Cw1) was done by isoelectric focussing. The autoradiography (Fig. 3) shows the pattern of bands expected for HLA-A2, B27 and Cw1 (Neefjes *et al.*, 1986; Güssow *et al.*, 1987). Only in the case of Cw1 the anti-fusion protein-antibodies differed in pattern and intensity from HC10.

These data suggest that the antibodies recognize an HLA-class I-specific, monomorphic determinant on slightly denatured HLA-antigens. Since the anti-fusion protein monoclonal antibodies do not directly bind to cells and cannot recognize fully denatured class I molecules, but can precipitate class I molecules, they seem to recognize a slightly denatured form of class I antigens.

Cellular responses to fusion proteins

Helper-like responses were analyzed by measuring the induction of IL-2 secretion from PBL stimulated with fusion protein presented by autologous monocytes. Two fusion proteins were able to stimulate T-cells to secrete IL-2 (Table 2a). Some determinants

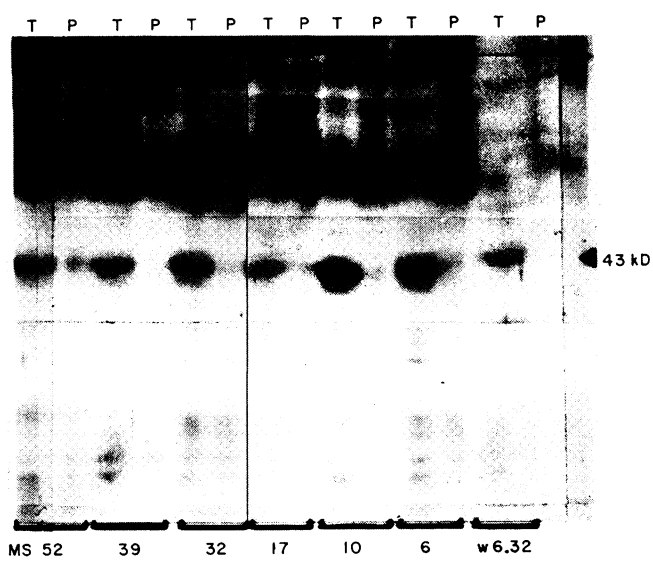

Fig. 2. Western blot. Isolation of B27 antigen from the mouse mastocytoma lines P815 (P) and P815/B27^{R3} (T) (P815 transfected with the B27-gene) with the monoclonal antibodies MS6, MS10, MS17, MS32, MS39, MS52 and W6/32. The blot was developed with the monoclonal antibody HC10. The lysates were prepared from 5×10^7 B27^{R3}-cells and 4×10^7 P815-cells.

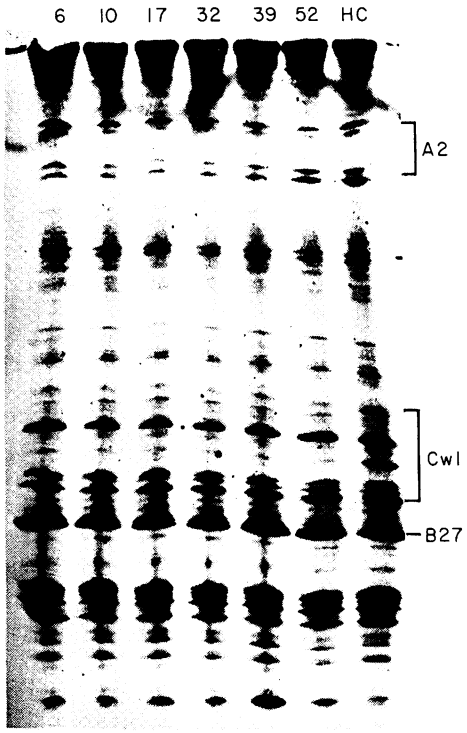

Fig. 3. Isoelectric focussing. Precipitation of HLA-class I-antigens from B-LCL LG2 with the monoclonal antibodies MS6, MS10, MS17, MS32, MS39, MS52 and HC10.

important for induction of IL-2-secretion may be located near residue 114 in the second domain (D1 and D2 are dissected at this site). Conformational determinants present in D1/2, however, may be more important, since IL-2 induction with this fusion protein approached levels induced by PHA stimulation of PBL.

To determine if the initial stimulation of the PBL with fusion proteins led to a specific priming for alloantigen (i.e. HLA-B27 specific), the same cultures were restimulated on day 10 with irradiated $B27^+$ and $B27^-$ PBL (Table 2b). Only those cells that were initially sensitized with fusion protein D1/2 produced high amounts of IL-2 in the secondary cultures. There may have been some preferential activation of T-cells specific for B27-associated allodeterminants, since the $B27^+$ PBL restimulated higher IL-2 secretion than the $B27^-$ PBL. The rapid decrease in IL-2 levels between 5 and 14 hr may occur because the primed responder T-cells themselves utilize IL-2.

Induction of CTL

The ability of the fusion protein D1/2 to induce CTL was tested by stimulating PBL of two different B27-negative donors with fusion protein β-galactosidase-D1/2 or β-galactosidase alone, presented either by autologous monocytes or $B27^-$ allogeneic LCL. Cytotoxicity was tested on $B27^+$ and $B27^-$ target LCL (Fig. 4). Effector cells from both donors showed much greater cytotoxicity of the $B27^+$ target cells than of the $B27^-$ targets, however this difference was also found with cells stimulated with β-galactosidase alone. Thus, there was no evidence for induction of B27-specific CTL by this fusion protein.

Table 2a. IL-2 secretion of T-cells activated by fusion proteins*

	18 hr	24 hr	48 hr
D1/2	51,315 ± 3317†	56,030 ± 4051	11,725 ± 4186
D1	396 ± 99	1056 ± 823	773 ± 265
D2	3799 ± 611	4012 ± 624	7691 ± 1093
D3	766 ± 279	695 ± 89	431 ± 15
MS2-Pol	708 ± 157	584 ± 72	569 ± 189

Controls: 5 units recombin. IL-2/ml: 71,356 ± 4491; 2,5 units recombin. IL-2/ml: 30,271 ± 1647; 1% PHA: 461 ± 137; RPMI-medium: 483 ± 169.

*Donor cells WG (HLA-A2, A3, B13, B44, Cw6) were stimulated with fusion protein pulsed autologous macrophages. 1×10^6 PBL were cocultivated with 5×10^5 fusion protein presenting macrophages in 2 ml RPMI-medium with 10% human serum. Culture supernatant was harvested after 18, 24 and 48 hours and tested for its content of IL-2 by ^{3}H-thymidine incorporation of an IL-2 dependent cell line.

†Data represent averages (± 1 S.D.) of triplicate values.

Table 2b. Restimulation of fusion protein-primed cells with $B27^+$ and $B27^-$ donor cells*

		D1/2	D1	D2	D3	MS2-Pol
$B27^+$ PBL	5 hr	10,825 ± 1370†	422 ± 71	580 ± 215	708 ± 212	333 ± 103
	14 hr	983 ± 352	1013 ± 150	824 ± 467	1319 ± 350	1865 ± 822
$B27^-$ PBL	5 hr	6488 ± 1093	580 ± 307	420 ± 117	480 ± 55	480 ± 208
	14 hr	496 ± 72	473 ± 51	643 ± 334	628 ± 208	628 ± 208

Controls: 5 units rec. IL-2/ml: 71,356 ± 4491; 2,5 units rec. IL-2/ml: 30,271 ± 1647; 1% PHA: 461 ± 137; RPMI-medium: 483 ± 169.

*$B27^+$ PBL EL: HLA-A2, B8, B27, Cw2, Cw7, DR3, DRw13, DQw1, DQw2. $B27^-$ PBL ST: HLA-A1, A26, B8, B14, Cw7, Cw8, DR3, DR7.

Ten days after primary stimulation with fusion protein presenting macrophages (see Table 2), PBL WG (HLA-A2, A3, B13, B44, Cw6) were restimulated with the same number of $B27^+$ (EL) and $B27^-$ (ST) PBL (10^6 cells/ml). After 5, 14, 18, 24 and 36 hr culture supernatant was collected and the content of IL-2 was determined. The table shows only the data from 5 and 14 hr supernatants, since at later times no significant responses were detected.

†Data represent average (± 1 S.D.) of triplicate values.

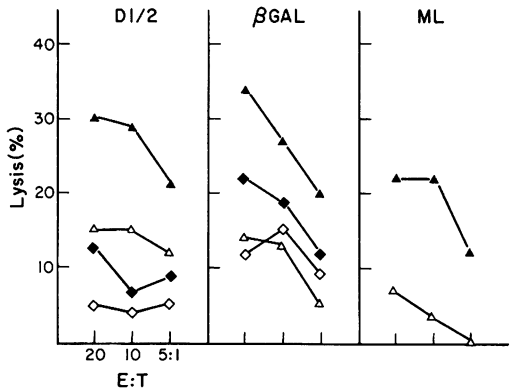

Fig. 4. Cytotoxicity of fusion protein prestimulated donor cells. Effector cells E1 (HLA-A2, B18, B60, Cw3, Cw8) and E2 (HLA-A2, A3, B35, B37, Cw4) were stimulated with autologous macrophages pulsed with fusion proteins D1/2-β-galactosidase and β-galactosidase alone and with LCL ML (HLA-A2, A3, B7, B27, Cw2). Target cells were LCL T1 (HLA-A2, A3, B27, B35) and LCL T2 (HLA-A2, A3, B35, B37, Cw4) as B27-negative control. ▲ Effectors E1 tested on targets T1 ($B27^+$); △ Effectors E1 tested on targets T2 ($B27^-$); ◆ Effectors E2 tested on targets T1 ($B27^+$); ◇ Effectors E2 tested on targets T2 ($B27^-$).

Inhibition of CTL

The fusion proteins were also tested for their abilities to inhibit target cell lysis. Effector cells were stimulated with $B27^+$ PBL and propagated for 21 days in the presence of IL-2. The CTL line was then analyzed for lysis of a target cell (HLA-A2, B27, Cw1) that differed only by B27 from the effector cells (HLA-A2, B17, B51, Cw1, Cw6). Varying amounts of fusion proteins were added to this CML combination (Fig. 5). Inhibition of up to 50% was found using 100 μg/well of MS2-fusion proteins. Again, all fusion proteins, including the control fraction of MS2 alone, inhibited lysis. Therefore, as with the induction of effector cells, it seemed that some bacterial protein non-specifically influenced the cytotoxic T-cell response.

DISCUSSION

Elucidation of the nature of the epitopes on alloantigens that elicit various immune responses has been studied thus far in two principal ways. Exon shuffling between different alleles was used to create new hybrid molecules, whose antigenicity for cytotoxic T-cells could be investigated (Ozato *et al.*, 1983; Arnold *et al.*, 1984, 1985). These experiments revealed that residues in the first and second domains, as well as conformation dependent epitopes are recognized by CTL. Similar results were obtained by comparing natural recombinant HLA-A genes in man (Clayberger *et al.*, 1985). Alternatively, CTL have been identified that can distinguish among variant class I molecules and sequence comparisons enabled identification of the antigenic regions important for CTL recognition. This approach revealed that residues 77–80 and 152–156 seem to be critical for alloantigen recognition by both human (Lopez de Castro *et al.*, 1985; Parham *et al.*, 1987) and mouse (Nathenson *et al.*, 1986) CTL. Peptides covering these amino acid stretches could also be recognized by cytotoxic T-cells (Walker *et al.*, 1985; Maryanski *et al.*, 1986; Parham *et al.*, 1987). While the results of the first approach point to the necessity of conformation, the restricted amino acid differences found in the second case imply an important role for short peptide sequences although how recognition of these stretches is dependent on conformation remains undefined. Fusion proteins offer a third way to study T-cell recognition of discrete regions of an HLA molecule. Since it is difficult to determine *a priori* the peptide regions antigenic for B- or T-cells, fusion proteins would seem to provide an ideal tool since sequences larger than peptides, nevertheless much more restricted in size than complete HLA molecules, can be easily generated. These longer sequences may also attain partial folding necessary for immune recognition.

As the binding studies to fusion proteins show, the monoclonal antibodies do not recognize determinants that are specific for B27. Except for antibody

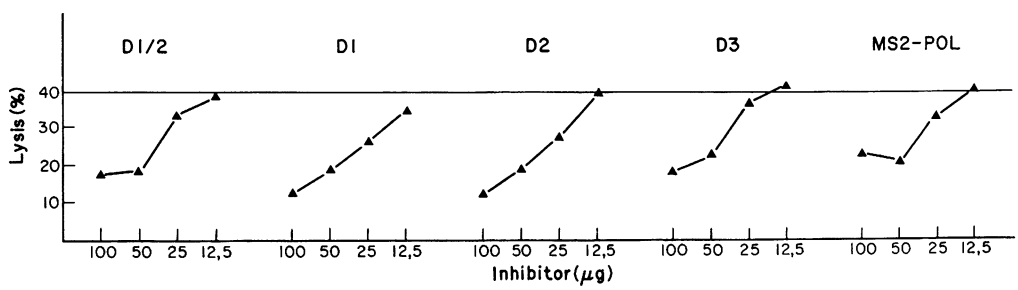

Fig. 5. Inhibition of B27-specific cytotoxicity with MS2-polymerase-fusion proteins. The T-cell WM (HLA-A2, B17, B51, Cw1, Cw6) was stimulated with PBL Has (HLA-A2, A3, B27, B37, Cw1). 21 days later cytotoxicity was tested on the target LCL LG2 (A2, B27, Cw1) with an effector:target ratio of 5:1. The solid line symbolizes the specific lysis without inhibitor; the concentrations of inhibitor (fusion proteins) are given in μg/200 μl microculture.

MS39 that obviously recognizes an unidentified bacterial product present in all fusion proteins and MS52 that is specific for an epitope in the third domain, the other monoclonals bind to determinants present on the first, second and third domains. Although D1 and D3 share no common amino acid sequences, similar three-dimensional structures may be formed that are recognized by the monoclonal antibodies. The specificity of the MS-antibodies for class I was shown by precipitation of HLA-antigens followed by Western blotting and isoelectric focussing. The precipitation of HLA-A2, -B27 and -Cw1 from cellular lysates of the HLA-homozygous LCL LG2 means either that the antibodies recognize a monomorphic determinant on class I molecules or that complexes of class I antigens in the lysate lead to coprecipitation. At least the data show that the anti-fusion protein antibodies are specific for HLA-class I, as demonstrated by comparison with the binding patterns of W6/32 and HC10.

According to Kourilsky and Claverie (1986), cytotoxic T-cells should recognize their antigens as peptides in the same way as T-helper cells. In these studies fusion proteins were used as antigens to analyze both T-cell subsets. Using macrophages as antigen presenting cells it is expected that antigenic peptides could be processed from fusion proteins. Stimulation of T-helper cells to secrete IL-2 showed that the responding cells recognized fusion protein D1/2, but not D1 or D2 alone. It is likely that one antigenic determinant is located in the region around amino acid position 114, where D1 and D2 are dissected. Restimulation with PBL of $B27^+$ and $B27^-$ donor cells resulted in a significantly higher secondary response to B27-positive cells by those PBL initially primed to D1/2, thus for T-helper cells the fusion proteins can obviously function as HLA-analogues. Cytotoxic T-cells seemed to be stimulated by an unknown antigen present in all fractions of fusion proteins. Since, however, cytotoxicity was greater for $B27^+$ cells it raises an interesting point regarding MHC and disease association with respect to HLA-B27, since crossreactivity between bacterial antigens and MHC antigen has been proposed as one mechanism resulting in development of Ankylosing Spondylitis in $B27^+$ individuals (Schwimmbeck *et al.*, 1987; Chen *et al.*, 1987).

The failure to clearly detect HLA-B27 specific responses raises a number of questions about the applicability of fusion proteins for studying alloantigens. A major problem arises with the denatured form of the fusion proteins which are precipitated as inclusion bodies in bacterial cells. The purification procedures enabling one to obtain suitable material for cellular assays cannot exclude contamination with bacterial proteins which may give rise to non-specific immune responses.

It must also be considered that the bacterial part of the fusion protein (β-galactosidase or MS2-polymerase) may interact with the B27 sequence, such that important determinants of B27 are covered or altered in structure. It may also be that the bacterial partners alter processing so that appropriate allopeptides could not be generated. These problems will only be solved with new generation vectors that enable isolation of recombinant molecules in soluble and native form and enable easy removal of the bacterial part of the molecule, for example through specific protease digestion.

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