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Expression and immunogenicity of HLA-B27 in high-transfection recipient P815: a new method to induce monoclonal antibodies directed against HLA-B27

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The immunization of a (BALB/c x C57BL/6) F1 mouse with murine transfectants expressing the HLA-B27 antigen resulted in a panel of polymorphic monoclonal antibodies with specificity for HLA-B27 and some additional HLA-antigens. Specificity of the antibodies was defined by cytofluorometric analysis on a panel of lymphoblastoid cell lines (LCL) derived from HLA typed individuals. Three of these antibodies are cytotoxic, and one of them inhibits B27-specific T cell cytotoxicity. Our results indicate that HLA-class I transfectants could be used to generate polymorphic antibodies, and that these antibodies may be helpful for HLA typing and for definition of epitopes recognized by T cells.

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The transfection of isolated genes into recipient cells offers a new way to study the expression and biological function of the gene products isolated from their normal environment (Heyes et al. 1986, Barbosa et al. 1984, Maryanski et al. 1985, Cowan et al. 1985, Gomard et al. 1986, Herman et al. 1983, Mentzer et al. 1986, van de Rijn et al. 1984). Owing to its high association to rheumatoid diseases such as ankylosing spondylitis and Reiter's syndrome (Tiwari & Terasaki 1985, Terasaki 1980), high interest focuses on the HLA-B27 antigen. The gene has been isolated, sequenced (Weiss et al. 1985, Szöts et al. 1986) and transfected into the mouse mastocytoma line P815. Serological assays demonstrated that these transfectants express functional B27 antigens (W.K., unpublished observation).

These transfectants were used as immunogens to generate specific monoclonal antibodies. After immunization of mice with one high expressing transfectant clone, established with an *in vitro* mutated HLA-B27 gene, four monoclonal antibodies were isolated with specificity for B27 and additional HLA antigens (such as B8, B13, B15, B18, B37 and B44), according to indirect immunofluorescence analysis. One monoclonal antibody recognized all tested cells expressing HLA-class I

antigens. Three of these antibodies show complement dependent cytolysis, and therefore may be useful reagents for HLA typing. One of the monoclonal antibodies also inhibits B27 specific T cell cytotoxicity.

Material and Methods

Transfection of the mutated and unmutated HLA-B27 genes

The cosmid clone cd2.6 encoding HLA-B27^w (Weiss et al. 1985, Szöts et al. 1986) or a 6.5 kilobase Eco RI subclone (Kuon et al. 1986) were transfected into the mouse mastocytoma line P815 (Weiss et al. 1985, Van Pel et al. 1985). The Eco RI subclone contained a mutated B27 gene, generated *in vitro* by site directed mutagenesis, exchanging serine at position 131 to arginine. The transfection of the unmutated B27^w gene resulted in a transfectant clone designated B27.3, whereas transfectant clone B27.R3 expressed the mutated gene. After recloning, the transfectants were tested on a panel of monoclonal antibodies to control surface expression and specificity of the introduced HLA genes.

Immunization and fusion

A (BALB/c x C57BL/6) F1 mouse was immunized with the P815 (DBA/2) transfectant B27.R3 expressing the mutated B27 antigen with an amino acid exchange at position 131 from serine to arginine. 7×10^6 B27.R3 cells were injected intra-peritoneally together with 10^8 Bordetella pertussis (Behring Institut, Marburg), followed by a second injection with 1.2×10^7 B27.R3 cells and 10^8 B. pert. on day 59. Three days later the spleen cells were fused with the mouse myeloma X63Ag8.653 (Kearney et al. 1979, Galfre et al. 1977).

Cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS, Sebio, Boehringer) or 5% Clex (Falcon), 2 mM L-glutamine, 100 IU penicillin/ml, 100 µg streptomycin/ml, 20 µM 2-mercaptoethanol, 1 mM sodiumpyruvate and 1 mM non-essential amino acids at 37°C in a humidity-saturated atmosphere with 5% CO_2.

Supernatants were screened in an indirect immunofluorescence assay on a homozygous B27^w LCL (LG-2, Gatti & Leibold 1979) and the HLA-class I surface negative cell line Daudi (Ploegh et al. 1979). Positive hybridomas were cloned by limiting dilution before expansion.

Indirect immunofluorescence assay

Cells were pelleted in round bottom microtiterplates and incubated with antibody containing culture supernatant for 1 h. After washing with phosphate buffered saline (PBS), cells were stained with FITC-coupled goat anti mouse IgG & IgM (Dianova, Hamburg) for 30 min, washed, fixed with 1% paraformaldehyde and analyzed by fluorescence-microscopy or by cytofluorography (EPICS V, Coulter or FACScan, Becton and Dickinson). All steps were carried out on ice.

Complement dependent microlymphocytotoxicity assay

Complement dependent cytotoxicity of monoclonal antibodies was tested in a two-stage microlymphocytotoxicity assay on peripheral blood lymphocytes (PBL) according to Terasaki & McClelland (1964). For cytolysis we used rabbit complement obtained from Behring Institute, Marburg. Lympholysis in percent was calculated for a 1:10 dilution of supernatants. Lymphocytotoxicity was regarded as positive when at least 40% of cells were lysed.

Inhibition of cell mediated lymphocytotoxicity (CML)

PBL of donor MIWI (A2, B17, B51, Cw1, Cw6) were stimulated with PBL of donor

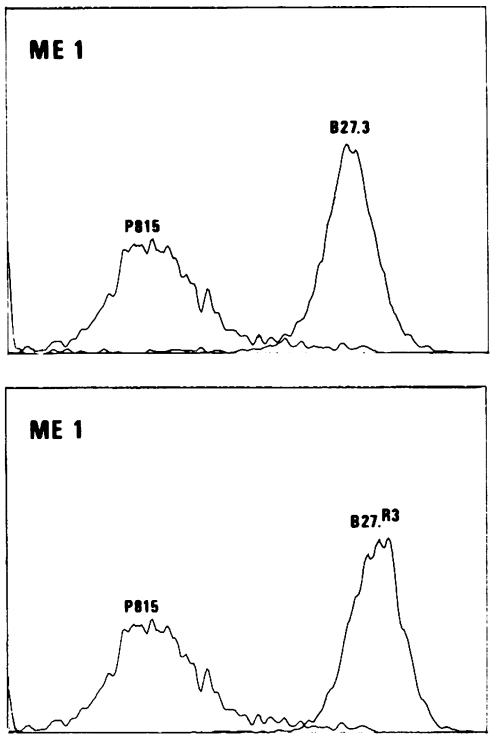

Figure 1. EPICS analysis: HLA-B27 surface antigen expression of P815 transfection clones (top: unmutated gene B27.3; bottom: mutated B27 gene B27.R3), determined by monoclonal antibody ME1 (specific for HLA-B27, -B7, -B22) (Ellis et al. 1982). Results are plotted as cell number (y-axis) versus log fluorescence intensity (x-axis).

HASE (A2, A3, $B27^W$, B37, Cw1) as described elsewhere (Schendel et al. 1978, Wildner et al. 1988). After 6 days, B27-specific cytotoxicity was tested in a 4 h chromium release assay on ^{51}Cr labeled LCL LG-2 (A2, $B27^W$, Cw1) in 200 µl of culture medium containing hybridoma supernatants of varying dilutions. Chromium release was measured and calculated as described by Schendel et al. (1978).

For secondary response, cytotoxic T lymphocytes (CTL) were maintained for 21 days with IL-2 but without further antigenic stimulation. In this case the chromium release assay was performed at the effector:target ratio of 5:1.

Results

Characterization of transfectants

Fluorescence profiles (Figure 1) show that both transfectants B27.3 and B27.R3 are recognized by antibody ME1 (Ellis et al. 1982), which is specific for B7, B22 and B27. This pattern was confirmed by the monoclonal antibodies W6/32 (Barnstable et al. 1978), B27M1 (Grumet et al. 1981) and B27M2 (Grumet et al. 1982), whereas non-related antibodies did not bind to the transfectants (data not shown). Figure 1 also shows that the B27.R3 transfectant P815 clone with the mutated HLA-B27 gene expressed an increased amount of surface HLA-B27 antigen compared to the transfectant B27.3 containing the unmutated form. Transfectant B27.R3 was selected for immunization because of its clonal stability and increased expression of B27-surface antigen.

HLA specificity of generated antibodies

1010 hybridoma supernatants from two fusion experiments with the spleen cells of one single mouse were screened on a small informative panel of LCL with B27 positive and B27 negative cells. Five of them were selected for further investigation and tested on a panel of 62 B-cell lines from unrelated, HLA-typed donors. The correlation between HLA antigens and monoclonal antibody reactivities was evaluated by the coefficient of correlation (r) computed from 2 × 2 tables, and significance (P) evaluated with a X^2 test (Simons & Tait 1984). The results are shown in Table 1.

The monoclonal antibodies TM-1, TM-4, TM-5 and TM-6 all recognize $B27^+$ cells and show crossreactivity to other HLA-B, but not to HLA-A, -C or class II antigens. Antibody

Table 1.
Cytofluorometry. Reactivity and r values of TM-1, TM-3, TM-4, TM-5 and TM-6 on unrelated B cell lines.

Antibody	Recognized antigens	No. of cells tested	No. of cells reactive with antibody	r[a]	P[a]	Total No. of cells tested with antibody[b]
TM-1	B27	9	9	0.67	0.001	61
	B44	17	13	0.60	0.001	61
	B15	3	3	0.56	0.001	61
	B27, B44, B15	28	24	0.68	0.001	61
TM-3	HLA-class I (monomorphic)	59	59	1.0	0.001	59
TM-4	$B27^W$	10	9	0.87	0.001	62
	B15	1	1	1.00	n.s.	62
	$B27^W$, B15	11	10	0.84	0.001	62
TM-5	B13	3	3	0.54	0.001	62
	B27	8	8	0.70	0.001	62
	B37	2	2	0.43	0.001	62
	B15	3	3	0.58	0.001	62
	B13, B27, B15, B37	18	17	0.76	0.001	62
TM-6	B15	5	4	0.50	0.05	60
	B44	14	13	0.69	0.001	60
	B27	8	7	0.61	0.001	60
	B8	6	5	0.55	0.025	60
	B18	4	3	0.50	0.05	60
	B15, B44, B27, B8, B18	38	32	0.66	0.001	60

[a] For calculation of r (coefficient of correlation) and P (probability), the other HLA antigens indicated in this table were excluded.
[b] Total number of tested cells, bearing 17 different HLA-A, 31 different HLA-B and eight different HLA-C antigens.
n.s. not significant.

TM-1 also bound to 76% of the $B44^+$ and 37% of the $B15^+$ cells. Exon shuffling experiments with hybrids of B27 and B7 indicate that the antigenic determinant for TM-1 is located on the α1-domain of B27 involving amino acid residues 77 and 80 (Toubert et al. 1988).

TM-4 strongly bound to all $B27^{W+}$ cells, except for the subtype $B27^K$. Also, very few (two of 15) HLA-$B44^+$ and $B15^+$ cells were stained with this antibody. In 1981 Grumet already described a monoclonal antibody, B27M1, dividing the B27 antigen into its subtypes $B27^W$ and $B27^K$. TM-4 shows a similar reactivity with respect to B27 subtypes, but the pattern of crossreactivity is different. The monoclonal antibody B27M1 crossreacts with B47, but not with B44 or B15 (Grumet et al. 1981).

Antibody TM-5 reacted with B27 and, to a lesser extent, B13, B15 and B37. This binding pattern is, except for B15, similar to that of the monoclonal antibody BD.7 (Bourel et al. 1987), supporting evidence of a common epitope on these antigens.

TM-6 recognized all B27-positive cells, and crossreacted with B44 and B15, whereas

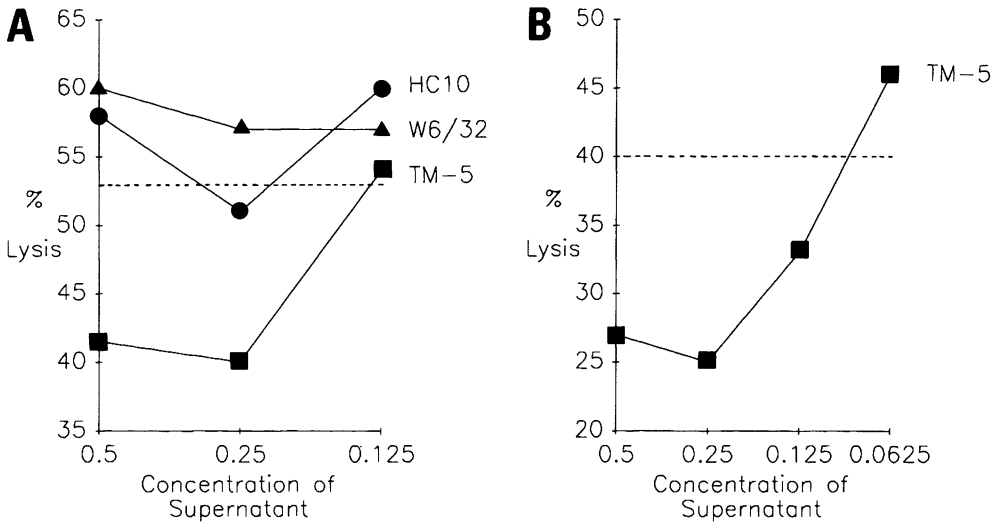

Figure 2. Inhibition of B27 specific cytotoxicity by monoclonal antibodies TM-5, W6/32 and HClO.

A: Inhibition of CML by monoclonal antibodies TM-5, W6/32 and HClO.
Effector MIWI: HLA-A2, B17, B51, Cw1, Cw6
Stimulator HASE: HLA-A2, A3, B27^w, B37, Cw1
Target LG-2: HLA-A2, B27^w, Cw1
Effector: target ratio = 15:1
4000 targets per well
Dotted horizontal line shows specific lysis without inhibitor (53%).

B: Inhibition of secondary CML of long term cultured CTL by monoclonal antibody TM-5.
Effector MIWI: HLA-A2, B17, B51, Cw1, Cw6 stimulated with IL-2
Stimulator HASE: HLA-A2, A3, B27^w, B37, Cw1
Target LG-2: HLA-A2, B27^w, Cw1
Effector: target ratio = 5:1
3000 targets per well
Dotted horizontal line shows specific lysis without inhibitor (40%).

TM-4 showed some crossreactivity with B8 and B18 bearing cells and recognized only some of these cells, although there are no serological splits of these antigens described so far (Terasaki 1988).

Antibody TM-3 bound to all HLA-positive cells. The binding-pattern was similar to that of W6/32 (Brodsky et al. 1979, Barnstable et al. 1978). Nevertheless, the experiments could not distinguish between the recognition of a HLA-B specific or a monomorphic HLA-class I determinant.

The monoclonal antibodies TM-1, TM-3, TM-4 and TM-5 did not show binding to the mouse mastocytoma P815, but, as expected, strongly reacted to a P815 transfectant expressing the modified antigen B27.R3 (TM-6 was not tested on transfectant cells).

Inhibition of CTL

Various concentrations of antibody containing supernatants were tested for their ability to inhibit the lytic activity of B27 specific cytotoxic T cells. Antibody TM-5 did inhibit cyto-

Table 2.
IgG-class and complement dependent cytotoxicity of monoclonal antibodies determined at a 1:10 dilution of culture supernatant. Lympholysis was regarded as positive if 40% or more of cells were lysed. + and − indicate positive or negative lympholysis.

Antibody	IgG-class	% lysis of target cells	
		LEER B8, B27	WLGE B13, B44
FCS		5−	5−
S43	IgG2a	5−	5−
W6/32	IgG2a	95+	95+
ME-1	IgG1	20−	15−
TM-1	IgG2b	95+	10−
TM-3	IgG2b	95+	95+
TM-4	IgG2b	15−	5−
TM-5	IgG2a	50+	30−
TM-6	IgM	5−	5−

toxicity of the primary response (Figure 2A), whereas W6/32 and HC10 (Stam et al. 1986) did not show inhibition as supernatants, although inhibition is described for W6/32 when used as diluted ascites fluid (Aparicio et al. 1985). Inhibition by TM-5 was confirmed in a second assay using the same, but long-term cultured effector line (Figure 2B).

Complement dependent cytotoxicity

Monoclonal antibodies were tested for cytotoxicity on $B27^+$ and $B27^-$ PBL (donor LEER: A2, B8, $B27^W$, Cw2, Cw7; and donor WLGE: A2, A3, B13, B44, Cw6) as target cells. Most of our undiluted supernatants were directly cytotoxic. For this reason, the lysis obtained at a 1:10 dilution of antibody containing supernatants is shown in Table 2. Further dilution of supernatants abrogated specific cytotoxicity. Table 2 also shows the Ig-isotype of the antibodies as determined by immunodiffusion (Ouchterlony 1970).

Negative controls with FCS, the unrelated antibody S43 (IgG2a, Reth et al. 1978) and the non-complement fixing antibody ME-1 (IgG1) (Ellis et al. 1982) lysed 20% or less of both target cells, whereas W6/32 (IgG2a) and TM-3 (IgG2b) showed strong lysis of the targets. TM-4 (IgG2b) and TM-6 (IgM) were not cytotoxic for these targets, although both antibodies recognized cells expressing the B27 or B44 antigen in indirect immunofluorescence. Antibodies TM-1 (IgG2b) and TM-5 (IgG2a) lysed the heterozygous $B8^+$ and $B27^+$ cell, but failed to lyse the $B13^+/B44^+$ PBL in this assay.

Discussion

Most commonly, PBL or Epstein-Barr virus transformed B cells are used as immunogens to generate monoclonal antibodies against HLA antigens. Since these cells present many foreign antigens to the mouse, the majority of obtained monoclonal antibodies is directed to unknown antigens on the B cell. Weak antigens hardly induce any antibody response. Murine transfectants carrying single HLA-genes offer the possibility of reducing the number of foreign antigens, especially in a syngeneic mouse system, and consequently may increase the yield of desired antibodies. Margulies et al. (1983) showed that syngeneic L-cells expressing a transfected alloantigen can induce antibodies specific for the transfected antigens in the serum of most mice. Heyes et al. (1986) described the generation of specific monoclonal antibodies by immunization with murine L cells transfected with class II antigens.

In our experiments, the mouse was immunized with a xenogeneic HLA-class I antigen on an H-2 compatible background. A (BALB/c x C57BL/6) F1 mouse with $H2^{d/b}$ was immunized with a P815 (derived from strain DBA/2 with $H2^d$) transfectant expressing the human MHC-antigen B27.

All antibodies presented in this paper are directed against the HLA-B27 antigen. In indirect immunofluorescence assay they all crossreact with additional antigens, preferen-

tially with B44 and B15. One antibody (TM-5) also recognizes to some extent B13 and B37, another (TM-6) B8 and B18. Antibody TM-4 did not bind to all cells carrying B44 or B15. Maybe it recognizes subtypes of these antigens, which are described elsewhere (Terasaki 1988).

Surprisingly, none of the common crossreactivities to HLA-B7, B22 or B47 were observed, although they are described for monoclonal antibodies as well as for alloantisera (Darke 1983). Initially, we discussed a possible change of immuno-dominant epitopes by the association with murine instead of human human β2-microglobulin. Nevertheless, presence of co-transfected human β2-microglobulin did not significantly alter expression of the class I antigens on transfectants (preliminary observation), although antibodies are available which differentiate human from murine β2-microglobulin in association with human class I antigens. The described TM-antibodies bound to transfectants with murine β2-microglobulin as well as to B-cell-lines with human β2-microglobulin. Moreover, different culture media supplemented with FCS or pooled human sera did not influence recognition of transfectants by CTL or generation of CTL by transfectants (W. Kuon, unpublished observation). Summing up, it may be said that the origin of β2-microglobulin seems to play a minor role in this special system. The mutation from Ser to Arg cannot explain the different crossreactivities, as B7 encodes Arg in position 131. One monomorphic antibody, TM-3, was obtained, which bound to all cells expressing HLA (-B) on their surface.

Antibodies TM-1, TM-3 and TM-5 were active in complement dependent microlymphocytotoxicity and thus may be useful for HLA typing. We observed some discrepancies between the reaction pattern when comparing the indirect immunofluorescence data with complement dependent cytotoxicity results. One explanation would be that LCL were used for immunofluorescence, which, compared to PBL, expressed an increased amount of HLA antigens.

Moreover, TM-5 could inhibit B27 specifc cell mediated lympholysis even at very low antibody concentrations (diluted culture supernatant), at which the antibody W6/32 did not show inhibition any more. This antibody may be helpful to map antigenic epitopes for T cells.

Having obtained these antibodies, we could demonstrate that transfectants can induce a humoral response in a xenogeneic system and may be very useful for the generation of serological reagents to cell-surface HLA class I-antigens. Owing to the mouse background of the transfectant cell, induction of antibodies to other human (MHC) antigens present on a normal HLA^+ human B cell can be avoided. Although in our case the mutation within the B27 gene did not alter the antigenic properties of the protein, transfectants may also be useful for generating serological reagents to genetically modified surface antigens.

Abbreviations

CML Cell mediated lymphocytotoxicity
CTL Cytotoxic T lymphocyte
FCS Fetal calf serum
HLA Human leucocyte antigen
LCL Lymphoblastoid cell line
MHC Major histocompatibility complex
FITC Fluoresceinisothiocyanate
PBL Peripheral blood lymphocytes
PBS Phosphate buffered saline

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