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Production of human monoclonal IgG antibodies against Rhesus (D) antigen

(human hybridoma/hemolytic disease of the newborn/Epstein–Barr virus)

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ABSTRACT **An Epstein–Barr virus (EBV)-transformed human B-cell line (LB_{4r}) producing anti-Rhesus [Rho(D) antigen] antibody was fused with a non-immunoglobulin-producing mouse–human heteromyeloma (SHM-D33) and selected in hypoxanthine/aminopterin/thymidine medium containing 0.5 μM ouabain. Surviving hybrids found to secrete specific anti-Rho(D) antibody were cloned by limiting dilution. Two clones (D4-B2 and E10-C1) producing high levels (12 and 20 μg/ml per 10^6 cells per 24 hr, respectively) of monospecific antibody (IgG3, λ chain) were selected for expansion and further characterization. Compared to the parental cell line (LB_{4r}), these hybridoma cell lines presented several advantages: antibody production was increased 10-fold, cloning efficiency was improved, and the EBV genome was not retained. Antibody production has been stable for >8 months. These human monoclonal anti-Rho(D) antibodies have demonstrated utility in routine blood-group typing. They may also prove useful in the biochemical and genetic characterization of the Rh antigen system. Most important, they offer a source of Rh-immune globulin for the prevention of Rh immunization and alloimmune hemolytic disease of the newborn.**

Since the introduction of postpartum administration of anti-Rh(D) human immunoglobulin to the Rh-negative unsensitized women who bear an Rh-positive infant, there has been a dramatic decrease in maternal isoimmunization and a decrease of Rh hemolytic disease of the newborn (1). However, as a result of the diminishing population of naturally sensitized serum donors and increasing demand for antenatal prophylaxis (2), new sources of human anti-Rh antibody are needed.

In the last few years, several methods have been developed to generate cell lines producing human monoclonal antibodies (mAbs). Somatic cell hybridization between specific B lymphocytes and mouse myeloma cells has been hampered by the difficulty of establishing stable cell lines in culture, mostly because of the selective loss of human chromosomes (3, 4). Sensitized B lymphocytes can be immortalized *in vitro* into B lymphoblastoid cell lines with Epstein–Barr virus (EBV) (5). However, such cultures usually secrete low levels of antibodies and have tended to cease production after a variable period (6). Human–human hybridomas secreting mAbs of predefined antigenic specificity have been described by several groups, including our own, but the yield of viable hybrids was too low for practical application (7–12).

Recently, a series of human–mouse heteromyeloma cell lines has been constructed and tested in our laboratory (13). One of these lines, SHM-D33, consistently generates a high yield of viable hybrids. In addition, the ouabain resistance of this line allows the selection of hybrids after fusion with lymphoblastoid cell lines that have been shown to give high yields in fusions with myeloma cell lines (5, 9). In this study, an anti-Rh antibody-producing lymphoblastoid cell line has been fused with SHM-D33 to generate numerous anti-Rho(D) antibody-producing hybrids.

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MATERIALS AND METHODS

Donor. Buffy-coat cells were obtained from a woman who had been sensitized to Rho(D) antigen during previous pregnancies. Her blood type was A Rhesus negative (Rh genotype: *cde/cde*) and her child was Rh positive (Rh genotype: *CDe/cde*). Two weeks after delivery, during routine postpartum examination and blood testing, a portion (20 ml) of the diagnostic specimen was collected in heparinized tubes. At that time, her anti-Rh antibody titer was 1024 (14).

Generation of Anti-Rh-Specific Lymphoblastoid Cell Lines. Peripheral blood mononuclear cells were obtained by Ficoll-Hypaque density-gradient centrifugation. T lymphocytes were depleted by mass rosetting with neuraminidase-treated sheep erythrocytes followed by a second Ficoll-Hypaque gradient to purify nonrosetted B lymphocytes. Purified B lymphocytes were transformed with the B95-8 strain of EBV as described (5). Emergent lymphoblastoid cells at a concentration of 2×10^6 cells per ml were mixed with an equal volume of a 4% solution of papain-treated O^+ human erythrocytes. The mixture was centrifuged at $800 \times g$ for 10 min and placed on ice for 1 hr. Then the cells were gently resuspended and applied to a Ficoll-Hypaque density gradient. The rosette-forming cells were recovered from the erythrocyte pellet after lysis of the erythrocytes with a solution containing 0.14 M NH_4Cl/0.01 M Tris·HCl, pH 7.4, for 10 min at 37°C.

Fusion Procedure. Lymphoblastoid cells were fused with the SHM-D33 heteromyeloma cell line as described (13). Selection for hybrid cells was effected in hypoxanthine/aminopterin/thymidine medium (15) with 0.5 μM ouabain. Hybrids producing Rh antigen-specific human Ig were cloned by two cycles of limiting dilution. For the generation of an antibody-rich ascites, hybridoma cells (5×10^6) were injected intraperitoneally into pristine-primed nude mice.

Screening for Rh-Reactive Human mAbs. Culture supernatants from wells exhibiting hybrid growth were initially screened for their ability to agglutinate papain-treated O^+ human erythrocytes and their reactivity in an anti-Rh(D) ELISA as described (16). The hemagglutination test was carried out by adding 80 μl of supernatant (at various dilutions) to 40 μl of a 2% solution of papain-treated human erythrocytes in round 0.2-ml wells and incubating at 37°C for 30 min. Positive supernatants were also treated by direct agglutina-

Abbreviations: mAb, monoclonal antibodies; EBV, Epstein-Barr virus; EBNA, Epstein–Barr nuclear antigen.

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tion in saline and by the indirect antiglobulin (Coombs) test (17). Each test was carried out in the presence and absence of dithiothreitol. The postpartum serum of the patient served as a positive control, and culture supernatant from the parental heteromyeloma cell line provided a negative control in all experiments.

Anti-Rho(D) Specificity Test. Supernatants giving a positive hemagglutination reaction with papain-treated O^+ human erythrocytes were subsequently tested in the same conditions with papain-treated O^- human erythrocytes. They were then tested against a panel of human erythrocytes of known blood group genotypes (Resolve Panel A, Ortho, High Wycombe) by the Coombs technique (17).

Characterization of the Hybridomas and Their Ig Products. Ig production was quantitated by ELISA (18) using heavy-chain specific goat anti-human Ig (Tago, Burlingame, CA) as well as by hemagglutination, with serial dilutions of culture supernatant. IgG subclasses were delineated using mAbs provided by Daniella Zelaschi (Department of Genetics, Stanford University, Stanford, CA). The hybrid nature and monoclonality of the hybridomas was established by Southern blot hybridization (19) using plasmid probes of the joining segment of the human Ig heavy chain genes and human λ light-chain constant region as described (20, 21). Newly synthesized immunoglobulins were labeled with [^{14}C]leucine and then sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out according to Laemmli (22).

Virus Studies. EBV nucleic acid hybridizations were carried out as described (22), using plasmids pDK14 (BamHI-V) and pDK225 (BamHI-K) (23) containing the IR1 large repeat and IR3 presumed Epstein–Barr nuclear antigen (EBNA) coding region of the B95-8 strain of EBV (24), respectively. Tests for EBNA were kindly performed by Werner Henle (Department of Virology, Children's Hospital, Philadelphia). Reverse transcriptase activity was assayed as described (25).

RESULTS

Derivation of Antigen-Specific Lymphoblastoid Cell Lines. Mononuclear cells from 20 ml of human donor peripheral blood were separated by Ficoll-Hypaque gradient centrifugation with the recovery of 2×10^7 cells. After mass rosetting with neuraminidase-treated sheep erythrocytes, a cellular fraction enriched for B lymphocytes (2×10^6 cells) was infected with EBV derived from the B95-8 marmoset cell line. Within 3 weeks, a polyclonal B lymphoblastoid proliferation was observed. The supernatant of the lymphoblastoid culture was tested by ELISA and hemagglutination for reactivity with O^+ human erythrocytes, with both assays yielding positive results.

An enriched population of anti-Rho(D) antibody-producing lymphoblastoid cells was selected by rosetting with papain-treated O^+ human erythrocytes. The rosette-forming cells were returned to culture and a repeat hemagglutination test confirmed their anti-Rh specificity (titer, 32). Lymphoblastoid cells (1×10^7) were then fused with SHM-D33 cells (5×10^6). In addition, the lymphoblastoid cell line was subcloned twice by limiting dilution. The mean level of anti-Rho(D) antibody production of the positive lymphoblastoid clones was 1.5 μg/ml per 10^6 cells per 24 hr.

Generation and Selection of the Hybridomas. The fused cells were selected in HAT medium containing 0.5 μM ouabain. After 2 weeks, proliferating hybrids were observed in 80% of the wells. All of the wells exhibiting hybrid growth contained human Ig in the culture supernatants by ELISA (22% IgG and 78% both IgG and IgM). Supernatant culture fluids tested for hemagglutination with papain-treated O^+ human erythrocytes were 100% positive. The level of Ig production ranged from 0.5 μg/ml to 20 μg/ml, with a mean production of 8 μg/ml per 10^6 cells per 24 hr. Twelve hybrids producing mAbs of the IgG isotype that gave the strongest hemagglutination reaction were subcloned by limiting dilution. More than 400 anti-Rh mAb-producing clones were obtained (all IgG, λ chain) and those with the highest level of Ig production were selected for establishment of permanent cell lines. The identification of human Ig heavy- and light-chain genes in the cell lines by Southern blot analysis confirmed their hybrid nature and monoclonality (data not shown).

The secreted mAbs, when tested in saline solution, did not show hemagglutination. However, as expected for an IgG, positive reactivity was observed by using indirect antiglobulin and enzyme techniques (both in the presence and in the absence of dithiothreitol). The IgG subclasses of the clones were determined by using murine monoclonal antibodies with specificity for human IgG subclasses, and all of the anti-Rh Ig-producing clones thus studied were found to secrete IgG3. Biosynthetic radiolabeling of the hybridomas with [^{14}C]leucine, followed by immunoprecipitation and polyacrylamide gel electrophoresis of the secreted Ig confirmed the production of human γ and λ chains (Fig. 1).

Specificity of the Anti-Rho(D) mAbs. The specificity of these mAbs for the Rho(D) antigen has been extensively analyzed by ELISA and hemagglutination techniques. As shown in Table 1, a panel of human erythrocytes of different blood groups in the ABO and Rh systems has been tested with the culture supernatants and has revealed the specificity of the mAbs for the Rhesus structure. The hybridoma supernatants were tested with human erythrocytes of different Rh genotypes and reacted only with erythrocytes that express the Rho(D) antigen (Table 2). The mAbs react strongly with all D-positive cells including those expressing the D^u antigen. When mAbs were tested in the Stanford University Medical Center Transfusion Service Laboratory against a panel of erythrocytes from ten thoroughly phenotyped donors, a clear cut anti-D specificity was identified, which was of comparable reactivity to commercial anti-D typing reagents. There was no reactivity with Duffy, Kidd, Lewis, MNS, Xg, P, or Lutheran antigens (L. Winn, personal communication).

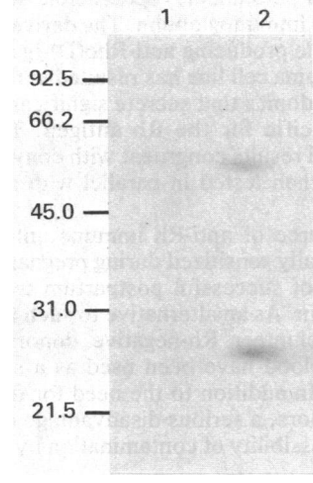

FIG. 1. Autoradiogram of sodium dodecyl sulfate/polyacrylamide electrophoresis gel, which shows the biosynthetically labeled human immunoglobin secreted by an anti-Rho(D)-specific hybridoma clone E10-C1 (lane 2), but not secreted by the heteromyeloma parent SHM-D33 (lane 1). Cultures of 10^6 cells were labeled with [^{14}C]leucine in leucine-deficient medium for 12 hr. Immunoglobulins were immunoprecipitated from supernatant fluids with affinity-purified goat anti-human γ-chain-specific antibody coupled with Affi-gel (Bio-Rad). Numbers indicate protein molecular weight standards × 10^{-3}

Table 1. Indirect hemagglutination reactivity with different blood group antigens

Blood group (Rh genotype)	Donor serum	LB_{4r}	D4-B2	E10-C1
A^+ (*R1 R1*)	+	+	+	+
A^- (*rr*)	−	−	−	−
B^+ (*R1 R1*)	+	+	+	+
B^- (*rr*)	+	−	−	−
AB^+ (*R2 R2*)	+	+	+	+
AB^- (*rr*)	+	−	−	−
O^+ (*R2 R2*)	+(1024)*	+(32)	+(256)	+(512)
O^- (*rr*)	−	−	−	−

*Anti-Rh hemagglutination titer performed with O^+ human erythrocytes only.

the established hybridoma cell lines have been compared with the parental cell lines SHM-D33 and LB_{4r}. As shown in Table 3, the growth rate was similar among the parental heteromyeloma cell line (SHM-D33) and the hybrids D4-B2 and E10-C1 derived from it, but these hybrids demonstrated a greater cloning efficiency than their lymphoblastoid parental line (LB_{4r}). The level of Ig production greatly increased after fusion and cloning. The two hybrids, D4-B2 and E10-C1, show Ig production, as measured by quantitative ELISA, of 12 and 20 μg/ml per 10^6 cells per 24 hr and anti-Rh hemagglutination titers of 1:256 and 1:512, respectively. In addition, both cloned hybridomas produced antibody-rich ascites on injection into nude mice. Analysis by molecular hybridization using EBV-specific probes (Fig. 2) and immunofluorescent staining for EBNA (data not shown) demonstrated that the majority of hybrids (10 of 12, including D4-B2 and E10-C1) had lost the EBV genome. Reverse transcriptase tests of the culture fluid supernatants were negative, suggesting that the cells do not produce murine C-type retroviruses.

DISCUSSION

In this report, we describe an *in vitro* system for the production of anti-Rho(D) human monoclonal antibodies in an attempt to provide a permanent, reproducible source of pure and highly specific immunoglobulin. The derivation of a lymphoblastoid cell line producing anti-Rho(D) Ig and its fusion with a heteromyeloma cell line has resulted in the generation of numerous hybridomas that secrete significant amounts of human mAbs specific for the Rh antigen. These human mAbs have yielded results congruent with conventional anti-Rho(D) antisera when tested in parallel with routine blood typing methods.

The current source of anti-Rh immune antisera, mostly from women naturally sensitized during pregnancy, is diminishing as a result of successful postpartum treatment with Rh-immune globulin. As an alternative to such naturally sensitized donors, volunteer Rh-negative donors immunized with Rh-positive blood have been used as a source of Rh-immune globulin. In addition to the need for frequent phlebotomies from donors, a serious disadvantage of human donor serum is the possibility of contamination by hepatitis and

Table 2. Indirect hemagglutination with a panel of different Rh genotypes

Anti-Rh sample	*R1 R1* (*CDe*)	*R1^w R1* (*C^{w}De*)	*R2 R2* (*cDE*)	R_or (*cDe*)	*r'r* (*Cde*)	*r''r* (*cdE*)	*rr* (*cde*)
Donor serum	++	++	++	++	−	−	−
D4-B2*	+++	++	++	+++	−	−	−
E10-C1*	+++	++	++	+++	−	−	−

*mAb at a concentration of 100 μg/ml.

Table 3. Characterization of hybrids and parental cell lines

Trait	Parental cell line		Monoclonal hybrid	
	LB_{4r}	SHM-D33	D4-B2	E10-C1
Doubling time, hr	24	36	38	30
Cloning efficiency at 1 cell per well, %	10	28	30	25
Presence of EBV	+	−	−	−
Ig production				
IgM	+	−	−	−
IgG	+	−	+	+
IgA	−	−	−	−
κ	+	−	−	−
λ	+	−	+	+
IgG subclass				
1	−	−	−	−
2	+	−	−	−
3	+	−	+	+
4	−	−	−	−
Ig production, μg/ml per 10^6 cells per day	1.5	0	12	20
α-Rh hemagglutination titer, 10^6 cells/ml per day	1:32	0	1:256	1:512
Growth in nude mice	No	Yes	Yes	Yes

cytomegalovirus (26), as well as the presumed infectious agent responsible for the acquired immune deficiency syndrome (AIDS) (27). Although sources of Rh-immune globulin are decreasing, the demand for it may actually be increasing. Antepartum prophylaxis with Rh IgG has been advocated in situations during pregnancy in which there is an increased risk of transplacental hemorrhage and Rh immunization, after amniocentesis, and after obstetrical procedures such as external cephalic version (2). Rh IgG must also be given to all Rho(D)-negative women after spontaneous or therapeutic abortion, and its provision is advocated for Rho(D)-negative women during the second trimester of preg-

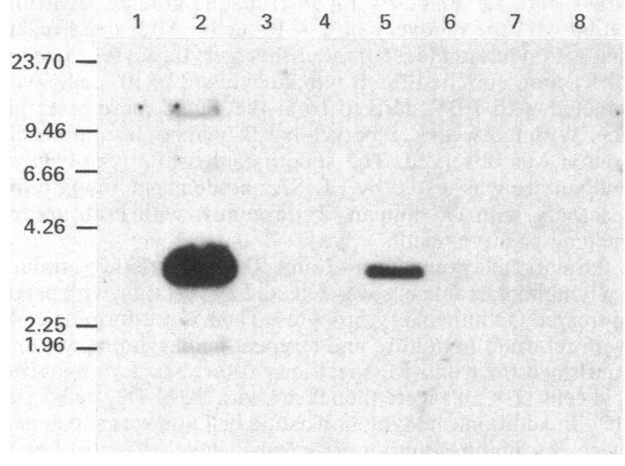

FIG. 2. Detection of EBV-specific sequences by Southern blot hybridization. DNA (10 μg) isolated from various cell lines was digested with *Bam*HI and fractionated through 0.7% agarose gels. After transfer to nitrocellulose, the filters were probed with nick-translated ^{32}P-labeled plasmid pDK14 (Bam V), which contains the IR_1 repeat of the B95-8 strain of EBV. Lane 1: SHM-D33, the heteromyeloma fusion partner cell line. Lane 2: LB_2, a representative lymphoblastoid cell line. Other cell lines: hybridoma clones, D4-B2 (lane 3), E10-C1 (lane 4), E10-C6 (lane 5), E6-1 (lane 6), and E6-2 (lane 7). Lane 8 is DHL-1, a human diffuse histiocytic lymphoma cell line. Numbers indicate the position of *Hin*dIII-cleaved bacteriophage λ molecular weight markers in kilobases.

nancy, as well as after delivery, to more effectively prevent Rh immunization (28).

Extensive screening of our anti-Rh mAbs has demonstrated their broad reactivity within the Rh system, recognizing all Rho(D)-positive human erythrocytes, including those expressing the D^u antigen. In addition to the advantages for typing and potential therapy that such broad reactivity affords, the identification of these mAbs as IgG3 antibodies further enhances their therapeutic potential. The efficacy of anti-Rh IgG in the prevention of hemolytic disease of the newborn has been clearly established, with IgG3 and IgG1 isotypes providing the most potent immune prophylaxis against Rh isoimmunization (29, 30). Thus, although our LB_{4r} was also producing IgM anti-Rh antibodies, as shown by a direct hemagglutination in saline only, we have concentrated our attention on IgG-producing hybrids, because there is general agreement that IgM does not prevent hemolytic disease of the newborn, and paradoxically, it may even enhance the sensitization against Rh antigen (30).

One of the primary concerns about the therapeutic use of antibodies produced by cell lines containing EBV or retroviruses is the possibility of viral contamination. In the present study, the negative reverse transcriptase analyses argue against the production of a murine retrovirus by the hybrid cells, while the EBV studies have shown that the majority of the hybrids do not retain the EBV genome. Although we have not been able to correlate the maintenance of the EBV genome with the presence of any specific human chromosomes in the hybrids, it can clearly be segregated from the genes coding for the structure and production of human Ig. This fact lends a major advantage to our system in comparison to previous reports of anti-Rh EBV-induced lymphoblastoid cell lines (31–33). Additional advantages can be found in the greatly augmented level of Ig production and the ability of the hybridomas to grow and produce an antibody-rich ascites in nude mice. Furthermore, in contrast to most lymphoblastoid cell lines, which are known to lose Ig production after several months, the Ig production of clones D4-B2 and E10-C1 has been stable for >8 months in continuous culture. Although an 8-month follow-up does not yet allow us to conclude that fusion increases the stability of Ig production, the fact that our hybrids are producing mAbs composed of IgG3 λ chains suggests that their chances of long-term stability are high. Indeed, it has been shown that the human chromosomes 14 (heavy-chain locus) and 22 (λ-chain locus) are preferentially retained in human–mouse hybrids, while chromosome 2 (κ-chain locus) is preferentially lost (34, 35). The molecular cloning of the Ig genes resident in our hybridomas offers the hope of providing a perpetual standardized source of Rh-immune globulin.

The procedure we describe for the production of human mAbs recognizing the Rho(D) antigen holds both analytical and therapeutic promise. These reagents have proven useful in blood-group typing. They may also facilitate a more definitive biochemical characterization of the Rh antigen structure and help to explain the genetics of the Rh system. The techniques described here may also be applied to generate human mAbs against the Rh antigens implicated in a minority of cases of alloimmune hemolytic disease of the newborn (anti-c, anti-E, anti-Kell, and others) and could also prove useful in studies of the erythrocyte antigen systems involved in the pathogenesis of the autoimmune hemolytic anemias.

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