Production of Specific Antibody Without Specific Immunization

R.A. Goldsby, B.A. Osborne, D. Suri, A. Mandel, J.Williams, E. Gronowicz, L.A. Herzenberg

Under appropriate conditions, the fraction of B lymphocytes stimulated in a population of mouse spleen cells may be large enough to constitute a representative sample of the total repertoire of antibody specificities which reside in the entire set of B lymphocytes (1). However the transitory nature of the polyclonal response (depending on culture condition, it declines and eventually ceases after 4-8 days) has prevented its exploitation as a strategy for the production of useful amounts of antibodies to particular antigens. The demonstration (2) that continuous cultures secreting antibody could be obtained by the hybridization of mouse myeloma and mouse spleen cells suggested a means of preserving the polyclonal response for an indefinite period of time. It seemed reasonable to assume that the hybridization of spleen cells in which a polyclonal response had been elicited by LPS with an appropriate myeloma cell line would result in continuous cultures displaying polyclonal antibody production. Using this approach, it was possible to isolate from the fusion of unimmunized (Balb/c x SJL)F_1 spleen cells with NS1, a nonsecreting, HAT sensitive variant of MOPC 21, hybrid populations in which the polyclonal response as evidenced by the elaboration of antibodies to human hemoglobin A, KLH, DNP and human RBC's was preserved (see Figure 1). Furthermore monospecific production of anti-DNP antibody was successfully factored out of the polyspecific production of antibodies by a hybrid population through the use of cloning. The expansion and subsequent injection of an active anti-DNP producing clone into a (Balb/c x SJL)F_1 mouse resulted in the formation of an antibody producing tumor. Collection of serum from an animal bearing such a tumor allowed us to obtain a useful quantity of an anti-DNP antiserum of high titer without resort to any program of immunization whatsoever (see Figures 2 and 3).

Hybrids were derived as follows: 2 x 10^8 LPS blasts obtained by the stimulation of spleen cells from 8-12 week old (Balb/c x SJL)F_1 female mice were mixed with 1 x 10^8 NS1 cells in 50 mls of serum-free Dulbecco's Modified Eagle's Medium (DME) and copelleted by centrifugation for 15 minutes at 400 g. The medium was removed and the pellet gently resuspended in 2 mls of PEG 1540 and incubated at 37° C for 1.5 minutes. During the 10 minutes following fusion, the suspension was gradually diluted up to 50 mls with serum-free DME and the cells harvested by centrifugation. The pellet was resuspended in 100 mls of HAT medium which had the following composition: 100 µM hypoxanthine, 10 µM aminopterin and 30 µM thymidine in DME containing 10% fetal calf serum, 2mM glutamine, 5mg/ml glucose, 100 units/ml penicillin and 100 units/ml streptomycin. After 48 hours of incubation at 37° C under an atmosphere of 90% air/ 10% CO_2 in mass culture, the cells were harvested by centrifugation and resuspended in 50 mls of HAT-DME. With the aid of a mechanical device for seeding, sampling and feeding cultures (3), 0.1 ml of the cell suspension was delivered into the wells of three 96 well microtiter plates onto a feeder layer of 2x 10^5 Balb/c thymocytes. The hybrid populations which appeared after 9-11 days were transferred by the mechanical replicator on day 14. Fluids from a replica were harvested, diluted three fold and tested for the presence of antibody to the antigens indicated in Figure 1.

Anti-DNP secreting clones were isolated from hybrid populations of a similar LPS x NS1 hybridization by the method of limiting dilution in microtiter plates, expanded to 10^6 cells and injected subcutaneously into 3-5 month old male (Balb/c x SJL)F1 mice. A tumor bearing mouse was exsanguinated and a portion of the excised tumor was returned to culture and another portion injected into a (Balb/c x SJL)F_1 recipient.

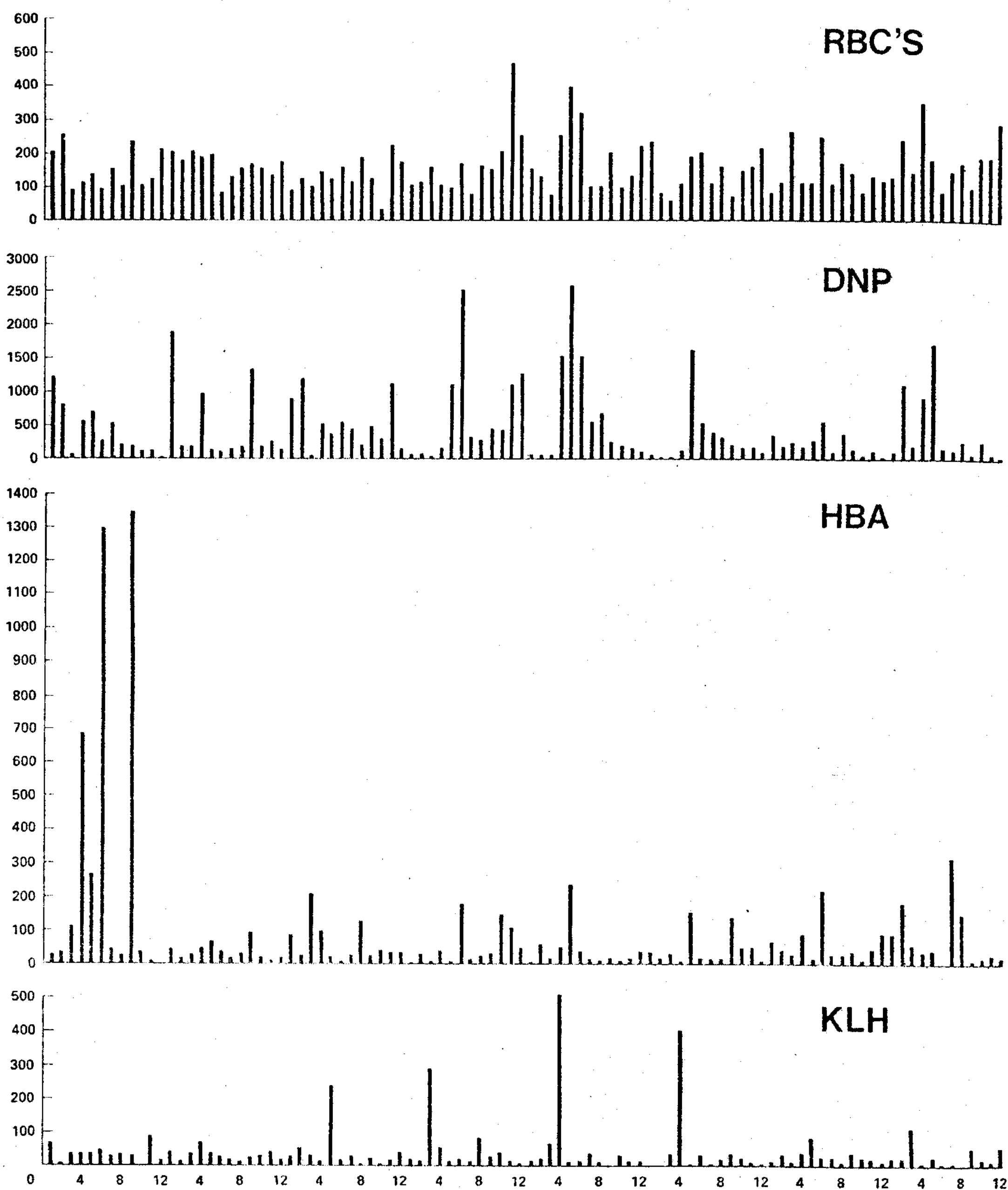

Figure 1. Preservation of the polyclonal response by hybridization. The fluids from 96 hybrid populations were assayed after fusion of 2×10^8 LPS blasts with 1×10^8 NS1 using 52% PEG 1540. The amount of antibody to the indicated antigen was determined by plate or cell binding radioimmunoassay using ^{125}I - labelled goat anti-mouse kappa chain antibody. It is clear that there is a spectrum of reactivity toward each of the antigens.

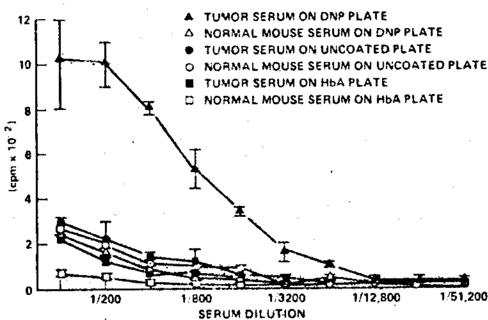

Figure 2. Antigen specificity of serum from an animal bearing an anti-DNP secreting tumor. Serum from an animal bearing a tumor raised by injection of an LPS x NSI anti-DNP producing hybridoma was examined for antibody to DNP or human hemoglobin A in a plate binding radioimmunassay using an ^{125}I-labelled goat anti-mouse kappa chain antibody as a second step. Only plates coated with DNP conjugated BSA and treated with dilutions of serum from tumor bearing animals bound significant amounts of mouse Ig.

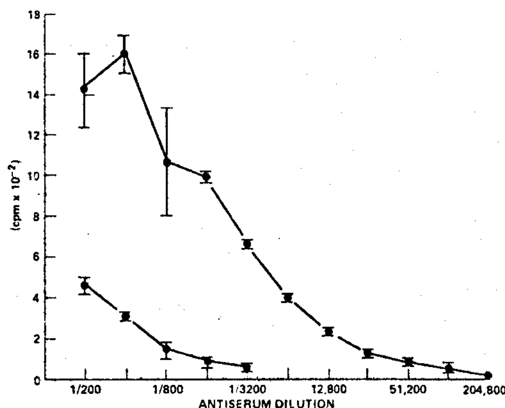

Figure 3. Reactivity of the anti-DNP heavy chain with an anti - μ. The hybridoma anti-DNP binds an ^{125}I-labelled rabbit anti-mouse u chain antibody, thus permitting the serological assignment of this antibody to the μ class. The upper curve represents the titration of the tumor serum on a DNP coated plate while the lower curve represents the normal mouse serum control.

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Clones of Human Lymphoblastoid Cell Lines Producing Antibody to Tetanus Toxoid

V.R. Zurawski, Jr., S.E. Spedden, P.H. Black, E. Haber

Introduction

We have previously reported (1,2) the establishment of a continuous rabbit cell line synthesizing and secreting antibody to type III pneumococcal polysaccharide. This line was established from a simian virus 40 (SV40) transformed spleen cell suspension obtained from a rabbit hyperimmunized with type III pneumococcal vaccine. This approach has not been generally applied because the quantity of antibody produced by the cell line, TRSC-1, was small (3) and resulted from an infrequently occurring transformation event. Furthermore, as reported in this volume by several laboratories, cell fusion methods (4) have regularly resulted in the establishment of murine hybridomas producing specific antibody.

Synthesis and secretion of immunoglobulin (Ig) by human lymphoblastoid cell lines is well documented (5). Therefore, to obtain cell lines synthesizing specific human antibodies, we have extended the viral transformation approach to human cells. We have effected viral transformation of human peripheral blood lymphocytes with Epstein-Barr virus (EBV), and demonstrated (6) that such EBV transformed human cell lines can be established that synthesize and secrete specific antibody to tetanus toxoid. Moreover, Steinitz et al (7) have independently reported establishing EBV transformed lines that produce antibody to another antigen, 4-hydroxy-3,5-dinitrophenacetic acid (NNP). This suggests that with appropriate selection of immunocompetent lymphocytes, the production of human antibodies to any antigen of interest might be accomplished utilizing EBV "immortalized" B cells.

Central to the problem of long-term antibody production by such cells is retention of their differentiated function despite the fact that they have been transformed to continuously proliferating lines. Our parent lines secreting antibody to tetanus toxoid did not retain their ability to secrete specific antibody after extended time in tissue culture (6). We report here the establishment of limiting dilution clones of these parent lines, which appear to be stable producers of antibody to tetanus toxoid.

Results and Discussion

Culturing cells at limiting dilution on feeder layers has proven to be the best method for obtaining clones of our lyphoblastoid cell lines producing antibody to tetanus toxoid. Critical to successful establishment of such clones has been the type of cells in the feeder layer. We found that human foreskin fibroblasts (HFF) provided an excellent monolayer that was stable for several weeks and evidently provided appropriate nutrients and/or growth factors to colony forming lymphoblasts. Human embryonic kidney, amnion, or embryonic lung cells were not adequate for this purpose. Placental cells (8) have not yet been evaluated.

Cells of the parent lines in log phase growth were suspended in RPMI 1640 medium, supplemented with 20% fetal calf serum and 2 mM glutamine. No doublets were detected in at least 5000 cells counted. Serial five- or tenfold dilutions of parent cell lines were made in the supplemented RPMI 1640 medium. Addition of conditioned medium did not seem to affect the efficiency of cloning. Aliquots of 100 µl of each dilution at 10^4 through 1 cell per well were placed in 96-well Costar microtiter plates containing confluent HFF monolayers. HFF cells at lower passage number (<20) were most effective as feeder layers. The peripheral wells of the microtiter plates were filled with phosphate buffered saline

(PBS), and were not used for culture, leaving 60 wells per plate containing cells. At least 10 plates were seeded for each cloning experiment. Inspection of microtiter plates indicated that lymphoblastoid colonies began appearing in those wells where only 1 cell had been seeded as early as two weeks after initiation of the cultures of most parent lines. After eight weeks, no new colonies were observed in any wells. Therefore, wells with viable cells were counted several times between two and eight weeks to determine approximate cloning efficiencies.

Table 1 presents cloning data for some parent lines at various passage levels. Cloning efficiencies for the lines were variable. Antibody to tetanus toxoid was detected in only a small percentage of the wells seeded at 1 cell per well. For some parent lines no antibody was found in wells so seeded, although at 10^5 or 10^4 cells per well, antibody was detectable (data not shown).

Table 1: Cloning efficiencies of various cell lines on HFF monolayers

Parent line	Passage	Cells seeded per well	Wells with lymphoblastoid colonies (%)	Wells with colonies producing antitetanus antibody (%)
3GC-C2(2H8)	5	100	100	N.D.
		10	82	5
		1	36	0.8
3GC-C2(F3)	1	10	100	8
		1	1	0
3GC-C2	6	1	3	2
3GC-C2	7	10	35	14
		1	5	0
3GC-C2	8	1	7	0
3GC-C5	5	100	6	0
		10	0	0
		1	0	0
4LP-C3	6	1000	92	0
		100	72	0
		10	17	0
		1	1	0
4LP-B4	8	10	100	N.D.
		1	13	N.D.
4LP-B4	9	10	100	N.D.
		1	83	2

When 4LP-B4 at passage 9 was seeded at 1 cell per well, 450 out of 540 wells (83%) contained lymphoblastoid colonies. Of these, probably no more than 38% were true clones, as computed from Poisson distribution curves. Only 7 of the 450 clones (2%) showed evidence of antitetanus antibody production. Six of these 7 were successfully established in continuous culture for more than five

months, and were routinely passaged for more than 10 weeks. For this entire time, they retained their ability to synthesize and secrete antibody to tetanus toxoid, achieving concentrations of 10 ng/ml in the culture supernatants, as determined by radioimmunometric assay (6). These antibodies also bound a highly purified sample of tetanus toxin (9). The stability of antitetanus antibody production by each of the six clones far exceeded that for the parent line 4LP-B4.

Cytoplasmic and surface staining for Ig light and heavy chains with specific fluoresceinated antibodies revealed that only λ light chains and γ heavy chains were being synthesized by each of the six clones whereas the parent line 4LP-B4, although synthesizing predominantly λ and γ chains, also was synthesizing μ and α heavy chains as well as κ light chains. Thus it is likely that the antitetanus antibody synthesized is of the IgG-λ isotype.

Recloning of the six clones producing antibody revealed a marked increase in the frequency of colonies, seeded at 1 cell per well, that produced antitetanus antibody. It has also served as an approximate measure of the clonotypic nature of the parent lines. For example, it can be seen (Table 2) that with recloning of clone 1E5, 15% of wells contained colonies that were secreting antibody. With clone 2F11, 87% of such wells contained detectable antitetanus antibody.

Table 2: Cloning efficiencies of clones 1E5 and 2F11 on HFF monolayers

Parent clone	Passage	Cells seeded per well	Wells with lymphoblastoid colonies (%)	Wells with colonies producing antitetanus antibody (%)
1E5	2	1000	100	100
		100	100	N.D.
		10	89	N.D.
		1	12	15
2F11	3	1000	100	100
		100	100	100
		10	89	100
		1	10	89

We have established clones of lymphoblastoid cell lines, which are evidently stable producers of antibody to tetanus toxoid. However, the true monoclonal nature of either these first or second generation clones must still be verified by thorough analysis of the secreted antibody. Monoclonal human IgG antibody to tetanus toxoid (toxin), synthesized in vitro and obtained in sufficiently large quantities, may prove useful clinically for passive immunization.

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