Production of Antibody to Tetanus Toxoid by Continuous Human Lymphoblastoid Cell Lines

Abstract. *Peripheral lymphocytes from human volunteers boosted with tetanus toxoid were cultured after in vitro infection with Epstein-Barr virus. Forty-four continuous lymphoblastoid lines were established which continued to secrete human gamma globulin; seven of these secreted antibody to tetanus toxoid. Subcultures derived from limiting dilution experiments continued to secrete the antibody. Some of these antibody-secreting cells have been in continuous culture for more than 6 months.*

Continuous lymphoid cell lines from several sources have been established, a number of which synthesize and secrete immunoglobulin (*1*). Among these sources are plasmacytomas (*2*) and lymphomas (*3*) of human origin. None of these, however, synthesizes antibody of a determined specificity. A previous attempt to initiate human lymphoblastoid cell lines in vitro capable of synthesizing specific antibody yielded lines synthesizing γ-globulin that was not of the desired specificity (*4*).

Workers in this laboratory (*5*) have shown that in vitro viral infection of cultures established from the spleen of a rabbit hyperimmunized with a bacterial vaccine could produce a continuous cell line synthesizing antibody with a specificity directed toward that vaccine. Köhler and Milstein (*6*) have successfully established murine lymphoid cell lines yielding antibody of predetermined specificity by fusing mouse myelomas with normal lymphocytes from immunized animals. This approach, producing specific mouse antibodies, has also been adopted in other laboratories (*7*).

We have attempted to produce continuous human cell lines yielding antibody of predetermined specificity. At least two continuous lines that are capable of long-term production of antibody to tetanus toxoid have been established from cultures of peripheral blood lymphocytes infected with Epstein-Barr virus.

Healthy adult human volunteers were each given one intramuscular booster immunization of tetanus toxoid (0.5 ml). Blood was drawn at intervals subsequent to this injection. The mononuclear cell fraction consisting of lymphocytes and monocytes was isolated on Ficoll-Isopaque (*8*). Cultures of these cells and of cells similarly isolated from the blood of unboosted individuals were established in plastic 24-well tissue culture plates. Culture medium consisted of RPMI 1640 supplemented with 15 percent fetal calf serum, 2 m*M* glutamine, and other additives (*9*). The average initial cell density was 6×10^6/ml. The production of antibody to tetanus toxoid in serum and cell culture was monitored by a solid-phase radioimmunometric assay (*10*). Briefly, wells of microtiter plates (Cooke soft plastic) were coated with tetanus toxoid, then with fetal calf serum. After the wells were washed, samples were applied and allowed to incubate. After another washing, ^{125}I-labeled and purified rabbit antiserum to human γ-globulin or human $(Fab')_2$ was applied to the wells and incubated. A third washing and drying procedure was followed by cutting and counting individual wells. Standard curves were derived by assaying dilutions of human antibody to the toxoid after the antibody was purified by affinity chromatography (*11*). The radioactivity (counts per minute) bound was in direct proportion to the amount of antibody present. Specificity of the assay for tetanus toxoid was determined routinely by addition of the toxoid to samples, which inhibited binding of antibody. Also, cell media from cultures known to be producing nonspecific human γ-globulin failed to give positive results in the assay. Furthermore, assays performed on plates coated only with fetal calf serum also failed to give positive results. Production of human γ-globulin by the cells was examined, with ^{125}I-labeled staphylococcal protein A (*12*) in a solid phase assay similar to that described above (*13*). Cell cultures and continuous lines were generally assayed 4 to 5 days after feeding or subculture.

Table 1. Production of antibody to toxoid by various 3GC cell lines at passage 3.

Cell line	Human γ-globulin* (ng/ml)	Antibody† to tetanus toxoid (ng/ml)
3GC-B1	3	0
3GC-B2	35	0
3GC-B3	23	0
3GC-B4	120	0
3GC-B5	5820	0
3GC-C1	3960	18
3GC-C2	1530	164
3GC-C3	2680	0
3GC-C4	209	14
3GC-C5	1600	19

*Detected by binding with ^{125}I-labeled protein A, which does not detect subgroup 3 immunoglobulin G; values are averages of triplicate samples. †Values of antibody production below 20 ng/ml were determined with triplicate undiluted and ten times concentrated samples of media; zero indicates < 0.1 ng/ml.

To some of the tissue culture wells, 0.2 ml of pooled Epstein-Barr virus of the B95-8 strain (*14*) was added, usually 24 hours after initiation of the culture. Multiplicity of virus to cell was approximately 0.01 with a virus preparation that contained 10^5 transforming doses per milliliter. The cells were maintained as stationary suspension cultures in a humid atmosphere of 5 percent CO_2 in air. After infection, cultures were fed two or three times each week by removing one-fourth to one-half the medium and replacing it with fresh medium. Proliferating cells from individual wells were passed to succeedingly larger volumes and established as continuous lines with routine passage at 1:1 dilution beyond passage 4 in 75- or 150-cm^2 tissue culture flasks.

Limiting dilution cloning experiments were performed on these continuous lines at several passage levels. Serial five- or tenfold dilutions containing 10^5 through 10 cells per milliliter were made either with fresh medium or one-half conditioned and one-half fresh medium. Cells were then cultured at concentrations of from 10^4 through one cell per well in Costar 96-well microtiter dishes without or with feeder layers of human embryonic kidney cells, human amnion cells, or human foreskin fibroblasts (*15*).

Individuals not recently immunized (> 2 years) with tetanus toxoid usually had serum concentrations of antibody to toxoid in a range of 0.01 to 0.1 mg/ml. Booster immunization produced ten- to 100-fold increases in the serum concentration of specific antibody. Most individuals achieved peak serum concentrations near 1 mg of antibody per milliliter within 2 weeks of immunization.

Cell cultures established from Ficoll-Isopaque fractionation of whole human blood of volunteers also were monitored for production of antibody to tetanus toxoid. With cells from "unboosted" individuals, quantities of the antibody secreted into culture media were generally very small or undetectable (< 1 ng/ml). In cultures of cells initiated from individuals 1 to 4 weeks subsequent to booster immunization, the antibody secreted into culture media ranged from 0.1 μg/ml to more than 1.0 μg/ml.

At the end of 1 month, no viable cells were detectable by trypan blue dye exclusion in cell cultures initiated from three boosted and three control individuals in 110 replicate samples to which no Epstein-Barr virus had been added. Epstein-Barr virus was added to 260 additional cultures established from 15 bleedings of six recently boosted individuals. After varying periods (3 to 8 weeks), significant cellular proliferation could be seen in many wells exposed to virus. Of

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these, 44 continuous lines (17 percent) were successfully subcultured. Establishment of long-term cultures was quite variable among cells derived from different persons. From 7 to 29 percent conversion was achieved, depending on the donor. These continuously proliferating cells were chiefly lymphoblastoid in appearance, and 92 to 99 percent contained the Epstein-Barr early intranuclear antigen (EBNA) as determined by immunofluorescence microscopy. Of the 44 cultures yielding continuous lines, seven secreted detectable antibody to tetanus toxoid in association with the burst of cell proliferation occurring from 3 to 8 weeks in culture. These seven were all obtained from cultures initiated from three bleedings of two individuals. Only three cultures out of seven producing antibody contained toxoid at the time of initiation and infection. Of 41 remaining wells containing toxoid (0.5 μg/ml), eight continuous lines were derived that never produced any detectable antibody to toxoid. All 44 lines secreted some human γ-globulin.

An example of comparative γ-globulin and specific antibody synthesis by a group of continuous lines from one volunteer is illustrated in Table 1. These lines at the third passage had been in culture for more than 2 months at the time of assay. Only lines 3GC-B5 and -C5 contained the toxoid in initial cultures. The production of γ-globulin and production of the antibody to the toxoid are not necessarily parallel. For example, whereas 3GC-B5 is secreting nearly 6 μg of γ-globulin per milliliter, no antibody is detectable. In contrast, more than 10 percent of the γ-globulin produced by 3GC-C2 was antibody to toxoid.

Three continuous lines with relatively high initial production of the antibody have been examined for more than 3 months in culture (beyond passage 5). All three have shown evidence of decreasing production with extended passage (Table 2). Although in each case antibody production to toxoid was detectable even beyond passage 20 (approximately 5 months in culture), initial secretion of 0.1 to 0.3 μg/ml in 4 to 5 days declined to levels in the range of 1 to 2 ng/ml. The total γ-globulin production, as detected by the protein A assay, did not decline as rapidly. For example, for 4LP-B4 even at passage 40 (more than 7 months in culture), γ-globulin (more than 0.2 μg/ml) was still detectable.

To determine whether or not the decrease observed in specific antibody production was due to more rapid proliferation of nonantibody secreting cells, the antibody-producing capacity of several subcultures from limiting dilution experiments was examined. From dilutions made above passage 30, with human embryonic kidney cells as a feeder layer, no subcultures producing significant amounts of antibody to toxoid were found in 194 wells with growing colonies. Below passage 24 of lines 4LP-B4 and 3GC-C2 with human embryonic kidney, human foreskin fibroblasts, or no feeder layers, 102 out of 916 wells with growing colonies produced antibody to tetanus toxoid in excess of the parent line at the passage of dilution. Many of these subcultures of the parent lines were reestablished in continuous culture (Table 3). Subcultures F3, 2H8, and 3B10 show particularly striking increases in antibody production when compared to data for the parent line shown in Table 2.

A second series of subcultures initiated at limiting dilution was carried out with several of the sublines illustrated in Table 3. Production of antibody in these subcultures was directly related to the number of initiating cells. For example, antibody production averaged 190 ± 30 ng/ml in 30 replicate wells initiated with 10^4 cells of subpassage 1D2, and 31 ± 2 ng/ml with 10^3 initial cells.

Human foreskin fibroblast monolayers have proved to be the best feeder layer. We have observed production of antibody to toxoid in culture wells with growing colonies which were established from as few as 10 cells per well on such monolayers.

Progressive loss of the antibody in cell culture media was observed in succeeding generations of the parent cell lines. A transient stimulus relatively early in culture, resulting in de novo synthesis of antibody in untransformed cells unable to be continuously passed in vitro, could be offered as a possible explanation for the initiation and eventual decline of the specific antibody synthesis observed. Such a stimulus might be provided by macrophage bound antigen, for example. We believe this explanation is unlikely, however, since several subcultures in limiting dilution experiments produced antibody in excess of the parent line. By passage 23, for example, dilutions of the original media were approximately 10^{14}. This means that subculture 3B10 (Table 3) is a 10^{16}-fold dilution of the original culture, maintained in vitro for 6 months or more and still secreting more than 1 μg of antibody per milliliter.

The more likely explanation for progressive loss of antibody production is that the parent lines in each case are multiclonal in nature, and that only a relatively small number of the transformed

Table 2. Production of antibody to tetanus toxoid by two cell lines.

Passage	Time in culture (months)	Human γ-globulin* (ng/ml)	Antibody† (ng/ml)
	Cell line 3GC-C2		
0	1.0		300
1	1.5		225
2	1.7	1630	200
3	2.1	1530	164
5	2.5	2220	25
6	3.1	1060	15‡
9	4.0	1960	9‡
15	4.2	1050	5‡
24	5.3	930	2 to 3‡
	Cell line 4LP-B4		
0	1.0		150
1	1.2		105
3	1.8		75
5	2.5		15§
7	2.7	446	20§
8	2.8	515	15§
9	3.0	348	18§
16	4.0	272	8§
18	4.4	271	2§
21	4.8	242	2§
27	5.6	198	1§
40	7.2	259	<0.1§

*Detected by binding with ^{125}I-labeled protein A. Values represent averages of triplicate samples. †Values represent averages of six to ten samples. Values below 25 ng/ml were determined with undiluted and ten times concentrated media. ‡The range of error about the mean is 10 to 20 percent. §The range of error about the mean is 10 to 50 percent.

Table 3. Production of antibody to tetanus toxoid by subculture of parent line 3GC-C2. The subcultures were established from at least tenfold dilutions of parent cultures and all at passage 10 or beyond, that is, past 3 months in culture for the parent line.

Subculture designation	Passage*	Initial cells in subculture (No.)	Initial† (ng/ml)	Later‡ (ng/ml)
1D2	10	16,000	54	103
1E3	10	1,600	130	26
1F2	10	16,000	101	<1
1G3	10	16,000	108	10–15
1H2	10	16,000	227	10–15
C5§	10	10,000	52	45
1D8	10	1,600	177	<1
1G5	10	1,600	273	60
1H7	10	1,600	156	25
E10	10	1,000	47	81
F3	10	1,000	178	648
2B1	13	9,700	39	<1
2D1	13	9,700	95	<1
2F2	13	9,700	48	47
2G1	13	9,700	51	60
2H8	13	970	456	
G11	23	17,000	94	<1
D6	23	17,000	38	22
3B10§	23	1,700	1550	37

*Passage of parent line at time of dilution. †Highest level of antibody achieved in microtiter well in which dilution was accomplished. ‡Data obtained two additional passages after limiting dilution subculture; the cells initially seeded in 0.1 ml have multiplied to approximately 1×10^6 cells per milliliter at a volume of 2 to 3 ml. §These subcultures were not grown on feeder layers, all others were grown on human embryonic kidney monolayers.

lymphocytes are producing antitetanus antibody. With each succeeding generation in vitro, the antibody-producing cells, apparently not those best adapted to the tissue culture conditions, constitute less and less of the total cell population. This is consistent with the limiting dilution data. It is also consistent with the multiclonal infective potential of Epstein-Barr virus (*16*).

We believe that we have established continuous lymphoid lines producing human antibody to tetanus toxoid in quantities that might make stable clones of these lines a source of specific human antibody. Since we have observed specific antibody in cell culture media obtained from microtiter wells initiated with as few as ten cells and since the cloning efficiency on human foreskin fibroblasts is relatively high, it seems likely that homogeneous, stable antibody-producing clones will shortly be available. This approach may lead to a general method for production of specific monoclonal human antibodies in vitro.

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B Lymphocyte Antigens in Sicca Syndrome

Abstract. *All individuals tested in this study with sicca syndrome, a human autoimmune disease, bear two immunologically distinct and genetically unrelated B lymphocyte antigens that appear similar to the immune response associated (Ia) antigens of the mouse. The genes coding for these two antigens are present in only 37 and 24 percent of normal controls. In animal models Ia antigen genes are closely linked to immune response genes. Our findings suggest that two such genes may be required for the development of sicca syndrome.*

Loci of the major histocompatability complex (MHC) of man, located on chromosome 6, code for a series of human lymphocyte antigens (HLA). The seriologically defined antigens, HLA-A and -B, are widely represented on many body tissues in addition to lymphocytes. The lymphocyte-defined HLA-D region antigens, typed by the mixed lymphocyte reaction, are distinct from the HLA-A and -B antigens. Additional genes of the MHC, some of which are closely linked to the HLA-D region, code for antigens restricted to lymphocytes bearing surface immunoglobulin and Fc-receptor (B cells). These structures appear analogous to the immune response associated (Ia) antigens of mice. In that species, the genes coding for Ia antigens map in the same region as immune response (Ir) genes. Antiserums have been developed which recognize human B cell Ia-like molecules. These allow more precise definition of these potentially immunoregulatory cell surface antigens (*1*,*2*).

The autoimmune disease, sicca syndrome, also known as Sjögren's syndrome, consists of dry mouth and dry eyes caused by immunologic destruction of the salivary and lacrimal glands. Patients with this disorder have rheumatoid factor and other circulating autoantibodies which suggests that a disordered immune response may play a role in the pathogenesis of this disease (*3*).

Sicca syndrome is associated with a specific histocompatability antigen, HLA-B8, which is expressed in 50 to 55 percent of patients but only 20 percent of controls (*4*). Two subsequent studies revealed stronger (69 percent), but not absolute, association of sicca syndrome with the HLA-DW3 allele, which is in linkage disequilibrium with HLA-B8 (*5*), and suggested that sicca syndrome is primarily associated with this lymphocyte-defined antigen rather than HLA-B8.

The availability of antiserums to Ia-like antigens makes it possible to ascertain which specificities are associated with sicca syndrome. We determined the B lymphocyte antigens in 24 patients (21 females and 3 males, ages 26 to 73) and 184 normal controls (96 females and 88 males, ages 18 to 51) (*6*). Three of the patients have rheumatoid arthritis and three have systemic lupus erythematosus in addition to sicca syndrome.

Immunoglobulin-bearing lymphocytes were prepared as described (*1*, *7*). The 60 antiserums used were obtained from multiparous women and absorbed with pooled platelets to remove HLA-A, -B,

Table 1. B lymphocyte antiserums which distinguish patients with sicca syndrome from normal controls.

Antiserum	Sicca syndrome		Normal controls		χ^2	P*
	Positive	Negative	Positive	Negative		
Ia-172	24	0	68	116	31.7	<.001
Ia-AGS	24	0	44	140	52.5	<.001
Ia-35	16	8	31	153	27.3	<.001
Ia-350	15	9	39	145	16.8	.002
Ia-590	14	10	37	147	14.8	.007
Ia-715	13	11	26	158	19.8	<.001

*Corrected for the number of antiserums tested.

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