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Transition of Phenotypic Dimorphism with Regard to Spontaneous Sister Chromatid Exchange in Epstein-Barr Virus-transformed Bloom's Syndrome Lymphoblastoid Cell Lines

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We recently established four lymphoblastoid cell lines (LCLs) by infecting the peripheral blood of four Japanese patients suffering from Bloom's syndrome (BS) with Epstein-Barr virus (EBV). During the course of propagating these cell lines, two of them exhibited dimorphism regarding spontaneous sister chromatid exchange (SCE), i.e., a mixed population consisted of cells with extremely high SCE levels characteristic of BS and cells with low SCE levels indistinguishable from that of normal control cells. On the other hand, the other two cell lines maintained a monomorphic population with high SCE levels at least until 30 weeks after EBV infection. The proportion of the cells with high SCE levels in the cell lines with dual phenotype declined as the population doubling numbers (PDN) increased with time and they became ultimately undetectable. The proportion of cells with low SCE levels at the time of EBV infection was estimated in one of these LCLs as 0.075% by extrapolating the linear regression of the logit for the proportion plotted against PDN. In view of the well-known stability of the monomorphic phenotype in representative BS LCLs during extended cultivation, together with the present observations on the dual phenotype, we conclude that the frequent establishment of BS LCLs exclusively with low spontaneous SCE levels is attributable to the various proportions of low-SCE cells existing *in vivo* in the B-lymphocytes pool of BS individuals and to the selective pressure against the high-SCE cells in *in vitro* cultures.

Key words: Bloom's syndrome — EBV-transformed lymphoblastoid cell — Proliferative advantage — Sister chromatid exchange — Somatic mosaicism

Bloom's syndrome (BS[5]) is a rare autosomal recessive hereditary disease characterized by growth deficiency, an unusual facial configuration, sun-sensitive facial erythema and telangiectasia, immunodeficiency and a greatly increased incidence of a variety of malignancies at a relatively young age.[1)] The patients suffering from this disorder have been described as predominantly being of Ashkenazi-Jewish ancestry, although the recent survey by German and Takebe has revealed 11 cytologically verified BS patients in Japan, giving this country the second largest BS population.[2)]

Somatic cells from BS patients exhibit chromosome instability including quadriradial configuration,[3)] a greatly increased frequency of sister chromatid exchanges (SCEs),[3)] an elevated spontaneous mutation rate[4)] and a delayed maturation of replicative intermediates of DNA.[5)] Recently, some, but not all, BS lymphoblastoid cell lines (LCLs) have been characterized as showing reduced activity and thermal instability of DNA ligase I.[6–8)]

The remarkable increase in the frequency of SCE has been noted with cultures of peripheral blood lymphocytes, bone marrow cells, fibroblast cells and LCLs transformed with Epstein-Barr virus (EBV).[3, 9)] German *et al.* found, however, that a minority of BS patients had two populations of phytohemagglutinin(PHA)-stimulated T-lymphocytes, in which cells with extremely high levels of SCE co-existed with cells with normally low levels.[10)] The proportion of cells with normal SCE levels varied between approximately 1% and 47%.[10)] In addition, LCLs exclusively with normal levels of spontaneous SCE have frequently been established from the peripheral blood of BS patients,[11–13)] probably reflecting the existence of low-SCE B-lymphocytes *in vivo*. Establishment of LCLs with typically high SCE levels from BS patients has been technically difficult and labor-intensive, and therefore only a few such LCLs exist.[14)] We recently had the opportunity to monitor the kinetics of dual populations concerning spontaneous SCE in two out of four high-SCE LCLs which were newly established from Japanese patients with BS. The proportive advantage, expressed as the relative increase in the logit for the fraction, was approximately 0.134 per population doubling for cells with low SCE levels in one of these LCLs with dualism.

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[5] Abbreviations: BS, Bloom's syndrome; SCE, sister chromatid exchanges; EBV, Epstein-Barr virus; PHA, phytohemagglutinin; FPG, fluorescence plus Giemsa; PDN, population doubling number.

MATERIALS AND METHODS

The four patients with BS from whom the LCLs have been derived are listed in Table I. They were mutually unrelated except that 100YuMat and 101SaMat were siblings.[15] With all of them the diagnosis had been confirmed cytogenetically,[15-17] and they have been included in the BS Registry.[1]

The LCL was established by infecting mononuclear cells from a few ml of the peripheral blood with the B95-8 strain of EBV, as described previously.[18] All cell lines were maintained in a rapidly growing state by daily adjustment of the cell density to $5\text{–}7\times10^5$ cells/ml and replacement of half of the culture medium. In order to avoid drift, the size of each culture after establishment was kept above 1.5×10^7 cells. The cell density was determined with a Coulter counter, type ZBI (Coulter Electronics, Inc., Hialeah, FL) (threshold 10 μm; amplification 0.125; aperture 1). The total cell number of cultures was estimated from the cell density and the total volume of the cell suspension. The population doubling number (PDN) was calculated by dividing the natural log of fold increase in the total cell number by ln2.

Metaphase preparations and differential staining of sister chromatids were performed according to the fluorescence plus Giemsa (FPG) technique, as described previously.[19, 20] A minimum of 50 metaphase cells in the second mitosis were counted for each determination. The metaphases were also classified into M-1, M-2, and M-3 according to the pattern of sister chromatid differentiation. Both chromatids stained dark in M-1, one chromatid stained dark and the other light in M-2, and both stained light in M-3.

RESULTS

Heterogeneity for spontaneous SCE levels in BS LCLs Four BS LCLs were successfully established by infecting with EBV the mononuclear cell preparations from four Japanese patients suffering from BS (Table I).[18] In all four LCLs the majority of cells showed a distinct increase in the level of spontaneous SCE. BSF05, however, exhibited a bimodal distribution of SCE levels on the initial examination at 12 weeks (PDN33) after EBV infection (Fig. 1A, the upper panel). Twenty-one cells

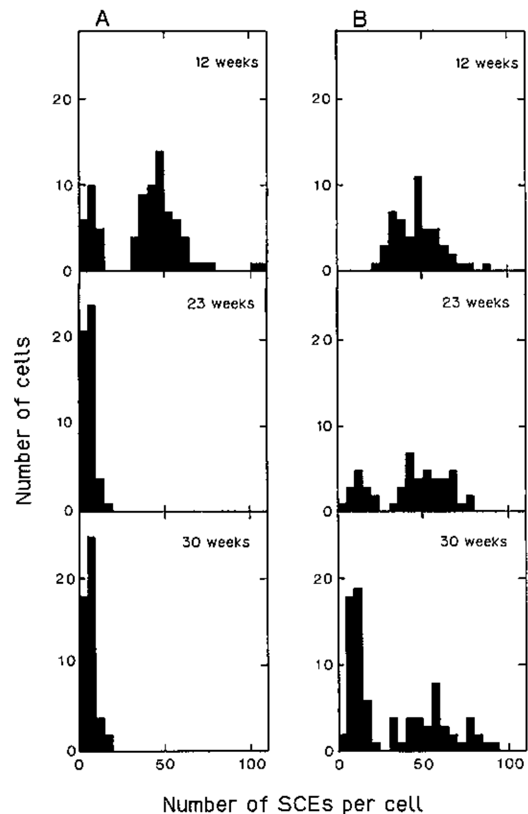

Fig. 1. Histograms of spontaneous SCEs in EBV-transformed BS lymphoblastoid cell lines. From 50 to 80 metaphase cells were subjected to SCE scoring for each determination. The histogram with class interval of 5/cell was constructed. (A) BSF05, (B) BSF06.

Table I. Origin of BS Lymphoblastoid Cell Lines

Cell line	Affected individual (BS registry#)	Age Sex[a]	Detection of T cells with low SCE (%)
BSF04	97AsOk (16)[b]	17 F	—
BSF05	86NoKi (17)	14 F	3/360 (0.84) (12)
BSF06	100YuMat (15)	11 F	1/1400 (0.07) (15)
BSF07	101SaMat (15)	10 F	6/1400 (0.43) (15)

a) Age, on sampling. Sex: F, female.
b) Reference number.

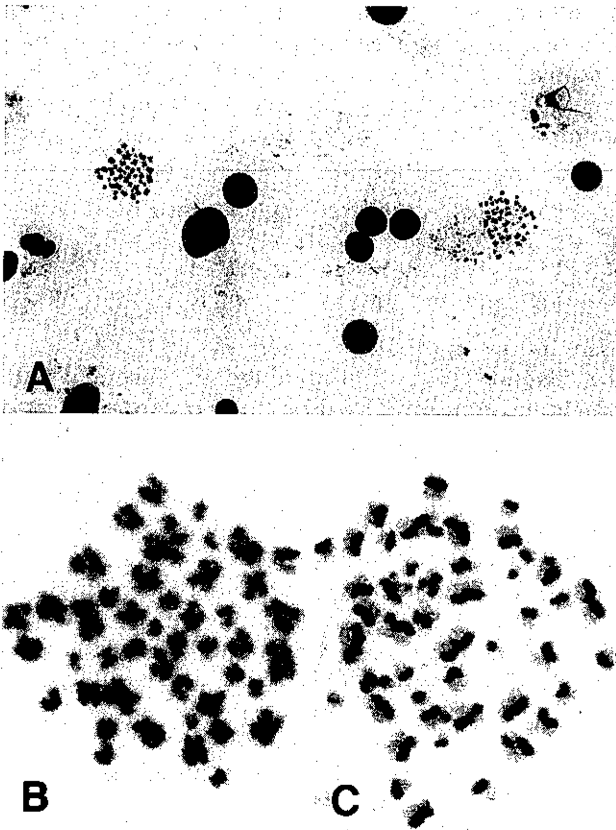

Fig. 2. Two adjacent metaphase karyograms with differential score of spontaneous SCEs prepared from BSF06 cells at 30 weeks after EBV infection. (A) Two karyograms representing phenotypic dimorphism in a low-power field (×80). (B) High-SCE and (C) low-SCE karyograms taken from (A) at a higher magnification (×320).

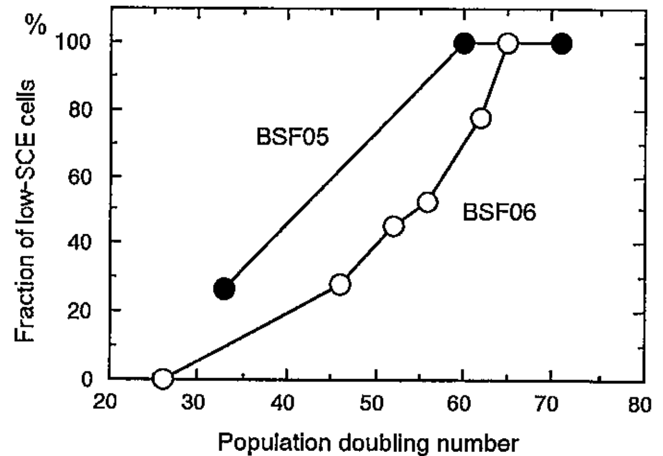

Fig. 3. Proportion of cells with low SCE as a function of population doubling number. Closed circles and open circles denote BSF05 and BSF06, respectively. The data are taken from Fig. 1 except for those at PDN 54, 62 and 65 of BSF06.

(26%) were found with a mean SCE level of 7.1±3.5 (1–14), which was in the normal range, while 59 cells (74%) exhibited a mean SCE per cell of 49.6±15.5 (32–115). The range of spontaneous SCE levels in the two populations did not appear to overlap. For another BS LCL, BSF06, cells with low SCE levels were not detected in 50 metaphase cells at 12 weeks after EBV infection (PDN26), and the SCE level per cell was 46.5±13.5 (24–85). Nonetheless, at 23 weeks (PDN46), 14 metaphase cells (28%) exhibited 3–21 SCE/cell (12.7±5.5) and 36 cells (72%), 31–75 (52.7±11.3) (Fig. 1B, the middle panel). Co-existence of the two types of cells with distinct SCE levels became more evident at 30 weeks (PDN 56), i.e., 42 cells (52%) were found with low SCE levels (10.2±4.2/cell) and 38 cells (48%) with high SCE levels (57.9±16.5/cell) (Fig. 2). No heterogeneity regarding SCE level was noted with BSF04 (82.6±16.4/cell) and BSF07 (128.8±19.4/cell), at least at PDN 32 and PDN 47, respectively. The karyogram for BSF07 on the first scoring of SCE demonstrated that essentially all cells were tetraploid, partly accounting for the very much higher SCE score per cell.

Population doubling times for BSF04, BSF05, BSF06 and BSF07 estimated from the slopes of growth curves were 74.7 h, 77.4 h, 103 h and 103 h, respectively. We did not detect any appreciable alteration in the doubling times associated with the transitions of SCE phenotypes in either of BSF05 and BSF06. The staining pattern of cells in these LCLs, reflecting the number of S-phases they had gone through in the presence of BrdU for 72 h, was also compared at the different culture ages of these LCLs. The proportions of BSF05 cells observed in M-1, M-2, and M-3 were 29%, 47%, 24% at PDN 33 and 45%, 41%, 14% at PDN 60, respectively. Those of BSF06 cells were 40%, 49%, 12% at PDN 26 and 38%, 47%, 17% at PDN 56. Thus, the occurrence of dichotomy into high-SCE and low-SCE subpopulations in these 4 LCLs was correlated with neither the number of population doublings nor the cell cycle traverse.

Ultimate predominance of low-SCE cells On the second examination of BSF05 at 23 weeks (PDN 60), the histogram for SCE per cell demonstrated a monodisperse distribution of 5.6±3.5 (n=50) (Fig. 1A, the middle panel), and the proportion of high-SCE cells, if present, should be lower than 1/50. We could not detect a high-SCE cell in 50 BSF05 cells at 30 weeks (PDN 71). The mean score was 6.2±3.2 (ranged 1–15) (Fig. 1A, the bottom panel).

The proportion of cells with low SCE levels in the BSF06 culture also increased with cultivation period, as shown in Fig. 3. At PDN 65, 38 weeks after EBV infection, the cells with high SCE levels appeared to have been annihilated from the culture.

Estimation of selective pressure for low-SCE cells in BSF06 culture Because we subjected only 50 to 80 metaphase cells to each SCE scoring, the fraction of cells with low SCE either below a few percent or above 90% could not be measured with statistical precision. For BSF06, however, we could monitor the change of the fractions in the range from 28% to 78% frequently enough to estimate the degree of fitness of low-SCE cells.

The number of high-SCE cells (N_1) at a given culture age, PDN (t), is expressed, by assuming exponential cell growth, as: $N_1 = b_1 \cdot \exp(a_1 t)$,
where a_1 and b_1 denote the apparent growth coefficient per population doubling of high-SCE cells and the number of high-SCE cells at the initiation of the culture, respectively. Likewise, the number of low-SCE cells at a given t is expressed as $N_2 = b_2 \cdot \exp(a_2 t)$. Then, the proportion of low-SCE cells (P) is expressed as follows:

$$\begin{aligned} P &= N_2/(N_1+N_2) \\ &= [b_2 \cdot \exp(a_2 t)]/[b_1 \cdot \exp(a_1 t) + b_2 \cdot \exp(a_2 t)] \\ &= 1/\{b_1/b_2 \cdot \exp[(a_1 - a_2)t] + 1\} \\ &= 1/\{\exp[(a_1 - a_2)t + \ln(b_1/b_2)] + 1\} \\ &= 1/[\exp(-at+b)+1], \end{aligned} \quad (1)$$

where $a = a_2 - a_1 > 0$ and $b = \ln(b_1/b_2)$.
The function (1) is called a logistic function.[21] Its straight line transform (logistic transformation; logit) is:

$$Z = \ln[P/(1-P)] = at - b, \quad (2)$$

so that, if the logit, Z, is plotted against PDN(t), the points will fall on a straight line, with a as the slope and b as the intercept. We applied this model to the observations of PDN and logit for the fraction of low-SCE cells of the BSF06 culture. The regression of logit values on PDN was computed using the observed data as shown in Table II. The regression equation was obtained as:

$$Z = 0.134t - 7.20 \quad (3)$$

with the Pearson's coefficient of determination, $R^2 = 0.970$ (Fig. 4).

Thus, the selective pressure, difined here as the relative increase of the logit for the fraction was estimated for low-SCE cells as 0.134 per population doubling in BSF06. The initial fraction of low-SCE cells at the beginning of the cultivation (P_0) could also be estimated by extrapolating the line back to $t=0$.

$$P_0 = 1/[\exp(-Z_0)+1] \quad (4)$$

Since the Z intercept (Z_0) was -7.20, we obtained P_0 as $7.47 \times 10^{-4} = 0.0747\%$ by using equation (4). If we could assume that the same slope holds true for BSF05 and make use of just one P value at PDN 33, the calculation based on the intercept would give $P_0 = 4.21 \times 10^{-3} = 0.421\%$.

Table II. Logistic Function of Fraction of Low-SCE Cells and Population Doubling Number in BSF06 Culture

PDN (t)	Fraction of low-SCE cells (P)	ln $[P/(1-P)]$ (Z)
46	0.28	−0.94
52	0.45	−0.20
56	0.48	0.10
62	0.78	1.27

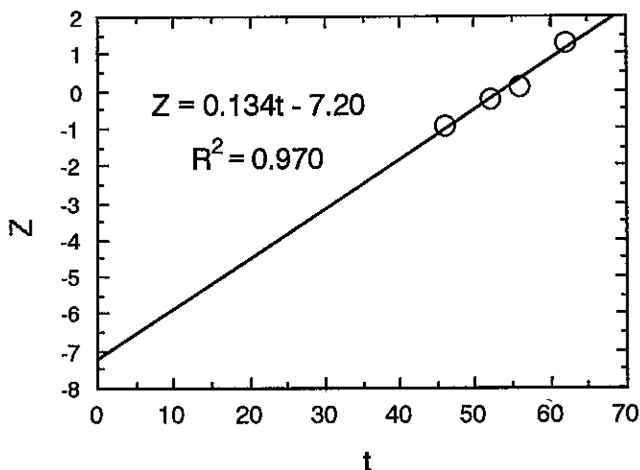

Fig. 4. Linear regression for the association between the logistic transformation of the fraction of cells with low SCE and the population doubling number in BSF06 culture. The data from Table II are plotted. The ordinate, logistic function of the fraction of cells with low SCEs, $\ln[P/(1-P)]$; the abscissa, population doubling number.

DISCUSSION

Occasional establishment of EBV-transformed BS LCLs which show exclusively normal spontaneous SCE levels[11–13] has attracted considerable attention, possibly because it is quite difficult to interpret in view of the autosomal recessive inheritance of the disease. In the present study, two of the four attempts to establish BS LCLs resulted in the emergence of a bimodal distribution regarding spontaneous SCE levels and an ultimate convergence to a monodisperse distribution of low-SCE cells. The fact that only some but not all of the BS LCLs

retained the characteristic phenotype of extremely increased SCE levels strongly implies great variability in the proportion of low-SCE cells in EBV-receptor-positive $CD21(CR2)^+$ B lymphocyte pools of BS patients.

The existence of a minor cell population, i.e., somatic mosaicism, has been well recognized for cultured lymphocytes and/or fibroblasts derived from persons with certain congenital anomalies[22] including another cancer-prone disease, xeroderma pigmentosum.[23] Areas of somatic mosaicism may result from the clonal expansion of variant cells that arise either from chromosomal abnormalities or from single-gene mutations during the course of embryonic, fetal, and extrauterine life.[22] Regardless of SCE phenotypes in the established LCLs, T lymphocytes with low SCE levels had been detected with 3 of the 4 patients at the frequencies ranging from 0.07% (100YuMat) to 0.84% (86NoKi) (Table I). The initial frequencies of low-SCE cells in the immortalized B cell pool, estimated for BSF05 and BSF06 by means of regression analyses of the proportion of low-SCE cells as a function of cultivation period, were in close agreement with the reported frequencies of low-SCE T cells in the corresponding PHA cultures.[12, 15] Thus, even though the co-existence of high-SCE and normally-low-SCE populations of cells in the blood has so far been detected only with cultures of PHA-stimulated T lymphocytes,[10, 12, 15, 24–27] we can reasonably presume low-SCE B lymphocytes also exist in the peripheral blood of at least some BS patients. Furthermore, it seems unlikely that any degree of preferential immortalization by EBV of low-SCE B cells plays a crucial role in the emergence of low-SCE BS LCLs.

Although essentially all human B lymphocytes possess EBV receptors (CD21/CR2), very few (usually less than 1%) are actually transformed into B LCLs after exposure to EBV.[28] Since we initiated the EBV-immortalization of BS B lymphocytes in mass cultures of approximately 3×10^6 cells in T-25 flasks, the number of transformed clones was expected to be 1,500 at most, assuming the proportion of B lymphocytes in peripheral mononuclear cells to be 5%.[29] The calculated frequency of low-SCE cells for BSF06 (7.47×10^{-4}) barely assures us of one low-SCE clone at the initiation of the culture. Hence, whether polyclonal BS LCLs contain a low-SCE cell clone or not should be determined from the Poisson distribution and would be affected by various factors including the frequency of low-SCE cells in B lymphocytes, the number of mononuclear cells at the initiation of culture, the proportion of B lymphocytes in mononuclear cells, the multiplicity of infection of EBV, the efficiency of transformation, and the occurrence of random drift during propagation. We consider it likely that a low-SCE cell clone was excluded by any of the above factors in the establishment of BSF04 and BSF07.

By employing the G-6-PD marker, it has been demonstrated that virtually all LCLs of polyclonal origin gradually evolve toward uniclonality within 12–18 months of continuous propagation.[30] This selection should occur at random since no preference for either of the two G-6-PD phenotypes is known. We believe, however, that the progressive selection in BS LCLs is clearly against the cells with high SCE levels, in view of the extremely high chance for the establishment of low-SCE BS LCLs as well as the transitions of SCE phenotypes in BSF05 and BSF06 in the present observations. For a low-SCE BS LCL, GM4408, the cells have been shown to lack the feature of a low level of DNA ligase I activity, whereas the abnormality is manifest for a fibroblast strain with high SCE levels from the same patient.[31] This association of the defect in DNA ligase I and the high-SCE phenotype may explain the proliferative advantage for the low-SCE BS cells. The satisfactorily frequent monitoring of population kinetics with BSF06 as to the fraction of low-SCE cells (Fig. 3) enabled us to estimate for the first time the magnitude of selective pressure for low-SCE cells per unit time (see "Results"). The degree of proliferative advantage for low-SCE cells was not enough to cause a detectable acceleration of the population doubling in BSF06 cells. Nonetheless, this marginal proliferative advantage seemed enough for low-SCE cells to take over by PDN 65 the BS LCL cultures in which the proportion of low-SCE cells was estimated as below 1% to start with.

In contrast, this kind of selective pressure for low-SCE cells did not appear to be applied to *in vivo* T lymphocyte pools in BS patients, who presented two populations with respect to the spontaneous SCE level, since blood samples taken from two such patients approximately 1 year apart did not show increases but rather decreases in the proportions of low-SCE cells in PHA cultures, i.e., 47% → 28% in 11IaTh and 39% → 15% in 12DeTh.[9] In addition, one BS T cell line, BST-SY_2, immortalized by adult T cell leukemia virus, was demonstrated to maintain during cultivation *in vitro* over 6 months a fairly constant proportion of low-SCE cells (30%) in a bimodal population.[32] The selective pressure for low-SCE cells may not operate in different types of cells with different proliferative kinetics.

The origin of the low-SCE phenotype in BS B lymphocytes that circulate in the peripheral blood of BS patients is not clear yet. In view of the elevated spontaneous mutation rate at the *HGPRT* locus in cultured BS fibroblasts, Warren *et al.* proposed the hypothesis of back-mutation at a *BS* locus as an interpretation for the lymphocytes with low SCE levels.[4] We consider, however, that a spontaneous true-reversion from *bl/bl* to *bl/+* in a clone of progenitor stem cells is unlikely, judging from our recent findings that BS lymphoblastoid

cells are in fact hypermutable for 6-thioguanine resistance but not for ouabain resistance, which is a marker specific for missense base substitutions (Tatsumi *et al.*, unpublished), and that virtually all of the 6-thioguanine-resistant mutants arose from large deletions, not from point mutations (Tachibana *et al.*, unpublished). An epigenetic mechanism or a second mutation at another locus may possibly have suppressed the manifestation of high spontaneous SCE levels.

In conclusion, the present observations support the idea that the frequent establishment of BS LCLs with normally low SCE levels is attributable to the somatic mosaicism in the B-lymphocyte pools *in vivo* and the proliferative advantage *in vitro* for the lymphoblastoid cells with low SCE levels. The use of a limiting dilution culture in microtiter plates, rather than an ordinary mass culture, should allow the establishment of BS LCLs with high SCE levels.

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