left, with progressively more distal segments to the right. A leaf segment with numerous developing somatic embryos is shown in Fig. 1B. Figure 1C shows a scanning electron micrograph of a well-developed embryo arising directly from the leaf surface. A light microscopic study of leaf cross sections showed that direct embryogenesis occurred in mesophyll tissue (Fig. 1D).

Embryos physically removed from the leaf surface and placed on SH medium without auxin germinated and formed individual plantlets. On medium with 30 μM dicamba they formed embryogenic calli from which many more embryos could be recovered.

We do not know the reason for the differential response of segments along the leaf. However, since the grass leaf grows from the base (*5*), a differentiation gradient exists, with cells at the extreme basal portion actively dividing and nondifferentiated while distal cells are less mitotically active and more differentiated. In our system there seems to be an optimum point on the differentiation gradient where direct embryogenesis is more frequent. This hypothesis would also account for the shifting of the embryogenic response toward the base of the older of the two leaves, since as leaves become older the meristematic area is restricted further toward the base. In fact, a response was not always obtained from the uppermost segments of the older leaf.

Wernicke *et al.* (*6*) observed dry, compact globules on cultured basal leaf segments of 10-day-old rice seedlings. They did not report whether these globules developed into embryos while still on the explant; only about 1 percent germinated normally when transferred to regeneration medium. Some showed a slight disorganized proliferation and multiple root and shoot formation.

In our experiments embryos formed in large numbers and grew to a highly organized stage. When embryos were isolated at the stage shown in Fig. 1C or later and placed on medium without auxin, more than 95 percent germinated into single plantlets that could be established in soil.

Mesophyll has been reported to be the most suitable tissue for protoplast research because relatively uniform protoplasts can be easily isolated in large numbers (*7, 8*). However, regeneration of whole plants from mesophyll protoplasts has yet to be accomplished in grass and cereal species. Our system may be of value in research efforts toward that goal. Formation of embryos directly from mesophyll cells may also provide an opportunity for in vitro cloning not previously possible because of tissue culture–induced variability (*9*). Although we have not yet conducted cytogenetic studies, none of the many plants that we have recovered exhibit chlorophyll deficiency or other phenotypic abnormalities indicative of tissue culture–induced variability.

The single-cell origin of the embryos has not been unequivocally established. However, the thin suspensor that forms and to which the embryos are delicately attached does suggest such an origin. The extensive discussion by Haccius (*10*) on the unicellular origin of nonzygotic embryos, including those that develop directly, further supports a single-cell origin.

Direct embryogenesis, in addition to its significance for plant tissue culture, provides a model system for studying the basic developmental morphology of nonzygotic embryos and the influence of auxins and other hormones on embryo development along a gradient of leaf segments. An understanding of the factors controlling this embryogenic response should advance the status of in vitro culture of mesophyll cells and protoplasts from a wide range of grass and cereal species and genotypes.

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Bloom's Syndrome: Evidence for an Increased Mutation Frequency in vivo

Abstract. *The incidence of lymphocytes resistant to the purine analog 6-thioguanine was studied in seven patients with Bloom's syndrome. The mean frequency was* 17.3×10^{-4}. *The mean incidence in age- and sex-matched controls was* 2.1×10^{-4}, *so approximately eight times the normal number of 6-thioguanine–resistant lymphocytes were detected in Bloom's syndrome blood. The basis for this increase is unknown, but the inherent genomic instability demonstrated in the form of chromosomal aberrations is one possible explanation.*

Bloom's syndrome (BS) is a rare autosomal recessive disorder characterized clinically by deficient growth, sun-sensitivity, immunodeficiency, and, in many cases, some form of cancer (*1*). The molecular basis of the syndrome is unknown, but experimental evidence (*2, 3*) points to an impairment of DNA replication. BS cells exhibit a striking degree of genomic instability as evidenced by greatly increased numbers of sister chromatid exchanges (SCE's), chromosomal aberrations, and chromatid interchanges between homologous chromosomes (*4, 5*). Cells with specific locus mutations have also been reported to be present in abnormally great numbers in BS fibroblast cultures. These include 6-thioguanine–resistant (6-TG^r) and diphtheria toxin–resistant cells (*6, 7*). We estimated the incidence of 6-TG^r variant lymphocytes in vivo by an autoradiographic method (*8*) for detecting T lymphocytes in the circulating blood that can undergo DNA synthesis in the presence of 6-TG. A significantly increased incidence of 6-TG^r lymphocytes was found in each of the seven BS patients examined.

The basis for the detection of 6-TG^r cells in this test system is as follows. Lymphocytes capable of proliferating in short-term culture in medium containing 6-TG are of two sorts: (i) noncycling cells responsive to phytohemagglutinin (PHA) and deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) (E.C. 2.4.2.8), which catalyzes the conversion of hypoxanthine and guanine to their respective nucleotides by way of the salvage pathway, and (ii) cells that are already in a proliferating cycle in vivo and which can proceed through at least one cell cycle in vitro before succumbing to the lethal effects of 6-TG. HPRT also can convert the purine analogs 8-azaguanine (8-AG) and 6-TG

Table 1. Spontaneous 6-TG^r lymphocytes in Bloom's syndrome.

Source of lymphocytes	With thioguanine			Without thioguanine			Variant frequency ($\times 10^{-4}$)	S-phase cells per 10^4 cells	Corrected variant frequency* ($\times 10^{-4}$)
	Labeled cells	Cells counted	Labeling index ($\times 10^{-5}$)	Labeled cells	Cells counted	Labeling index			
Controls									
1	2	79,018	2.531069	598	5000	0.1196	2.1163 ± 1.4970		2.1163
2	3	61,128	4.907735	882	5000	0.1764	2.7822 ± 1.6071	0	2.7822
3	2	76,994	2.597605	476	5000	0.0952	2.7286 ± 1.9316	1	1.7286
4	3	69,904	4.291600	712	5000	0.1424	3.0138 ± 1.7425	1	2.0138
5	3	134,767	2.226064	619	5000	0.1238	1.7981 ± 1.0394	0	1.7981
6	2	26,022	7.685804	878	5000	0.1756	4.3769 ± 3.0952	2	2.3769
7	2	88,344	2.263878	692	5000	0.1384	1.6358 ± 1.1570	0	1.6358
Mean							2.6360		2.0645
BS†									
9(EmSh)	21	152,371	13.782150	595	5000	0.1190	11.5816 ± 2.5374		11.5816
20(ViShr)	20	47,216	42.358523	798	5000	0.1596	26.5404 ± 5.9585	4	22.5404
7(RoTa)	12	28,487	42.124478	756	5000	0.1512	27.8601 ± 8.0515	3	24.8601
14(LeSi)	15	37,878	39.600824	750	5000	0.1500	26.4005 ± 6.8527	3	23.4005
15(MaRo)	10	43,946	22.755200	614	5000	0.1228	18.5303 ± 5.8820	3	15.5303
32(MiKo)	24	89,841	26.713861	679	5000	0.1358	19.6715 ± 4.0401	5	14.6715
59(FrFi)	16	121,410	13.178486	627	5000	0.1254	10.5092 ± 2.6422	2	8.5092
Mean							20.1562		17.2991

*Cycling S-phase cells were subtracted from the observed variant frequency. †Identified as in the Bloom's Syndrome Registry.

to their cytotoxic forms. Mutant cells lacking HPRT activity (HPRT$^-$ cells) are able to undergo DNA synthesis in the presence of normally toxic concentrations of 8-AG and 6-TG because the salvage pathway of such cells is nonfunctional, whereas normal, noncycling (HPRT$^+$) cells cannot survive. Thus HPRT$^-$ cells are able to incorporate [^{3}H]thymidine in the presence of 6-TG, making possible their detection by autoradiography.

Lymphocytes used to determine the incidence of circulating 6-TG^r cells were separated from heparinized blood on Ficoll-Hypaque gradients (Pharmacia). An age- and sex-matched control was analyzed alongside each BS patient. The method of Strauss and Albertini (*8*) [with minor modifications (*9*)] was used to determine the incidence of 6-TG^r lymphocytes.

The incidence of 6-TG^r lymphocytes in blood from healthy controls ranged from 1.6×10^{-4} to 2.8×10^{-4} (mean, 2.1×10^{-4}). The incidence in BS patients was eight times higher ($P < 0.001$) and ranged from 8.5×10^{-4} to 24.9×10^{-4} (mean, 17.3×10^{-4}) (Table 1).

All but one BS patient, 32(MiKo), were healthy at the time of blood sampling. Patient 32(MiKo) had been diagnosed as having acute lymphocytic leukemia 4 years earlier and was receiving maintenance chemotherapy in low dosage. In spite of this, the incidence of 6-TG^r lymphocytes in his circulating blood (14.7×10^{-4}) was similar to that in untreated BS patients. This was unexpected in view of the fact that Strauss and Albertini (*8*) had demonstrated an elevated incidence of 6-TG^r lymphocytes in cancer patients receiving chemotherapy.

Cells that become labeled with [^{3}H]thymidine in this test system are referred to as variant cells. An occasional mononuclear cell in normal human blood is engaged in DNA synthesis; the incidence of such cells increases in certain disease states and during certain phases of the normal response to antigenic challenge in normal persons (*10*). Such circulating, cycling cells appear to be resistant to the toxic effects of 6-TG (*11*) and may continue to replicate for sometime after being placed in 6-TG–containing medium. Such cells are indistinguishable from 6-TG^r mutant cells in the test system used here. Thus, cells referred to as variant in this autoradiographic test system comprise both true HPRT$^-$ mutants and nonmutant cells. In our study a rough estimate of the incidence of nonmutant cycling cells was made in the following way. Lymphocytes (2×10^5) were incubated at 37°C for 20 to 30 minutes in phosphate-buffered saline containing [^{3}H]thymidine (1 μCi/ml; specific activity, 75.5 Ci/mmole), fixed in methanol and acetic acid (3:1), and dropped onto microscope slides. These preparations were analyzed autoradiographically to determine the number of heavily labeled nuclei in 10^4 consecutive nuclei (Table 1). The frequency of cycling S-phase cells was then subtracted from the 6-TG^r variant frequency to obtain a corrected estimate (Table 1). In BS preparations the number of cycling cells was greater than in the controls, but the difference in variant frequency between the groups persisted whether or not the correction was made.

The basis for mutant detection in the test system used here is loss of HPRT activity. Some of the cells capable of synthesizing DNA in the presence of 6-TG presumably will have undergone a point mutation at the HPRT locus or lost a functional gene as a result of deletion or other chromosome mutation. (The HPRT locus is near the end of the long arm of the X chromosome at bands Xq26-27.) Cox and Masson (*12*) found microscopically detectable chromosome mutation to account for 40 percent of radiation-induced HPRT$^-$ mutations in human cell cultures. At this stage of our study of in vivo mutation in BS, the basis for the increased numbers of variant cells is unknown, but the cytogenetic anomalies in BS could give rise to multiple types of mutational events.

The 6-TG^r variant frequencies observed in control blood in this study (mean, 2.1×10^{-4}) are similar to those observed in an earlier study of 26 healthy individuals (*9*). [More recent data obtained with a cloning technique (*13*) that obviates the presence of cycling cells gave a mutation frequency of 1.2×10^{-5}, or in some cases as low as $\sim 5 \times 10^{-6}$. The discrepancy in the values obtained by autoradiographic and cloning techniques indicates that cycling cells provide a certain proportion of variant cells in the former method. Nevertheless, the differences between controls and BS patients studied here with the same technique under similar experimental conditions is striking.] The overall frequency is comparable to that expected (1.2×10^{-4}) if variants arise as a result of deletion of part or all of Xq (*9*). With regard to the increased incidence of 6-TG^r lymphocytes in BS, it is of interest

that chromosomal breakage is elevated in untreated cells. Shiraishi and Sandberg (*14*) reported that breakage in BS patients was ten times greater than that in normal persons (0.2 versus 0.02 break per cell). If one assumes that breakage sites are distributed among chromosomes in proportion to their lengths, then 1/40th of such sites would be on the X chromosome in male cells and 60 percent of these would be on Xq. Breaks affecting Xq in BS cells would be expected to occur with a frequency of 3×10^{-3}. Complete karyotypic analysis using G and Q banding of BS lymphocytes revealed six breakage sites in Xq among a total of 1023 metaphases examined (*15*), giving a frequency of 6×10^{-3}. The frequencies of 6-TG^r variant lymphocytes in BS in vivo (mean, 1.73×10^{-3}) are comparable to the expected incidence of breaks involving the long arm of the X chromosome. Thus it is possible that many of the 6-TG^r cells in BS arise through deletion or rearrangement affecting Xq. It will be interesting to determine whether point mutations also are elevated in BS in vivo.

A previously published observation that could be explained by an increased mutation frequency is the existence in some BS patients of two populations of lymphocytes with respect to SCE frequency (*16, 17*). Furthermore, lymphoblastoid cell lines, when developed from single BS lymphocytes isolated from the circulating blood, may exhibit either the characteristically elevated SCE frequency or a low (normal) frequency (*2*). In view of the elevated incidence of 6-TG^r lymphocytes in vivo, a plausible explanation for the presence in vivo of lymphocytes with a normal SCE frequency is back-mutation at the BS locus, as suggested by Warren *et al.* (*6*).

The true nature of the 6-TG^r variant lymphocytes that are increased in number in BS can be ascertained only by determining the heritability of the phenotype. The method used in the present investigation to detect 6-TG^r cells obviously precludes this because long-term cell growth of the suspected mutants is necessary. It recently has become possible, using T-cell growth factor, to clone human T lymphocytes and propagate them in vitro for prolonged periods (*18*). Using such a technique, Albertini *et al.* (*13*) were able to confirm the mutant nature of 6-TG^r lymphocytes. Therefore, such studies will be used to elucidate the biochemical and cytogenetic characteristics of the 6-TG^r lymphocytes circulating in the blood of persons with BS. Other loci can also be studied by using appropriate selective agents and the T-cell cloning technique to establish whether the BS gene does possess mutator activity when it is present in the homozygous state.

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Immune Response to Hepatitis B Surface Antigen: Enhancement by Prior Injection of Antibodies to the Idiotype

Abstract. *Anti-idiotype reagents that recognize a common idiotype associated with the combining site of antibodies to hepatitis B surface antigen (anti-HBs) were used to manipulate the immune response to hepatitis B surface antigen in BALB/c mice. The injection of antibodies to the idiotype before antigenic stimulation resulted in an increase in the number of cells secreting immunoglobulin M antibodies to hepatitis B surface antigen. Anti-HBs–secreting cells were also induced by administration of antibodies to the idiotype without subsequent antigen exposure. These findings indicate that the immune response to hepatitis B surface antigen in mice is regulated through an idiotype–anti-idiotype network.*

The immune response of an individual to a given antigen consists of a rapid recruitment of cells and soluble factors. Antigen-reactive T and B lymphocytes are partially regulated by cells that recognize their antigen-binding receptors. The basis for such regulation was postulated to involve a network of idiotype–anti-idiotype reactions (*1*). Idiotypes, located on or close to the antigen-binding site of both antibody molecules and lymphocyte antigen receptors, are the components of this network. Manipulation of the immune response by injection of antibodies to idiotypes has been documented in several systems [see (*2–14*); reviewed in (*15*)]. In these studies exposure to antigen after the injection of anti-idiotype reagents resulted in either suppression of the idiotype-positive antigen-binding molecules (*4, 5, 7*) or increased idiotype expression and antigen-binding activity (*2, 3, 8, 10, 12–14*).

Recently, we characterized a common idiotype shared by human antibodies to hepatitis B surface antigen (anti-HBs) (*16–18*). The common idiotype was associated in part with the antibody-combining site, because hepatitis B surface antigen (HBsAg) and a nondenatured HBsAg-derived viral polypeptide isolated in a micelle form partially inhibited the common idiotype–anti-idiotype reaction. This common idiotype was also expressed on anti-HBs produced in BALB/c mice and in six other species, indicating that an interspecies idiotypic cross-reaction was detected (*19*). We have now explored at the cellular level the regulatory consequences of in vivo administration of antibodies that recognize the common anti-HBs idiotype. Specifically, we analyzed the number of anti-HBs plaque-forming units (PFU) in the spleens of BALB/c mice previously immunized with antibodies to the idiotype.

Rabbit immunoglobulin G (IgG) anti-