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-

Table 1. Anti-HBs response expressed as PFU (mean ± standard error of the mean) per spleen. Each group of six mice received 40 μg of antibody to the idiotype or IgG from a nonimmunized rabbit (pre-IgG) or 5 μg of HBsAg on day 0, followed by the indicated injections on day 7, all by the intraperitoneal route. Spleens were removed on day 21. Cells from each mouse spleen were assayed in duplicate for the presence of both direct and indirect anti-HBs PFU. No hemolytic plaques were obtained with any spleen cells when uncoated or ovalbumin-coated SRBC were used as controls.

First injection	Second injection	Number of mice	Direct (IgM-secreting cells)	Indirect (IgG-secreting cells)
Antibody to idiotype	HBsAg	7*	531 ± 94†	178 ± 29
Pre-IgG	HBsAg	8	183 ± 65	125 ± 36
Antibody to idiotype	Antibody to idiotype	6	66.7 ± 47.8	100 ± 29
Pre-IgG	Pre-IgG	6	16.7 ± 16.7	8.3 ± 8.3
HBsAg	HBsAg	6	167 ± 36	1683 ± 168

*One mouse died during the experiment. †Statistical methods, including the two-tail Student's *t*-test and a single-factor analysis of variance by ranks test, the Kruskal-Wallis test (*27*), also indicated that direct PFU obtained in the group receiving antibody to idiotype before HBsAg was significantly different from all other groups of mice at $P = 0.05$.

serum to the idiotype was exhaustively adsorbed to remove isotypic and allotypic antibodies prior to affinity purification by acid elution from a CNBr-activated Sepharose 4B column coupled to the common human anti-HBs idiotype. The anti-idiotype nature of this rabbit antiserum has been described previously (*16*). An IgG fraction obtained from the serum of the rabbit before injection of the idiotype served as a control antibody preparation. We determined the antibody concentration of the two preparations in a spectrophotometer at 280 nm using an extinction coefficient of 15 for a 1 percent preparation. For the immunogen, we used nondenatured HBsAg-derived polypeptide, isolated as highly antigenic micelles (*20, 21*).

To quantitate the anti-HBs immune response at the cellular level, Jerne hemolytic plaque assays were performed in which IgM-secreting cells were measured by direct plaques and IgG-secreting cells by indirect plaques (*22*). Briefly, tannic acid–treated sheep red blood cells (SRBC) were coated with HBsAg (subtype *ayw*) and used as the indicator cells in the plaque assay. Passive hemagglutination was used with mouse anti-HBs to ensure the presence of HBsAg on the SRBC. Spleen cell suspensions from immunized BALB/c mice were mixed with HBsAg-coated SRBC in agarose. After incubation for 1 hour at 37°C, SRBC-adsorbed guinea pig complement was added. After 2 hours at 37°C, the direct hemolytic plaques due to secretion of IgM antibodies were counted and the number of PFU per spleen was calculated from the total number of cells per spleen. Indirect plaques due to IgG-secreting cells were determined by adding a serum containing rabbit antibodies to mouse IgG prior to the addition of complement. Both uncoated SRBC and tannic acid–treated SRBC coated with ovalbumin served as controls in each experiment.

To establish the time interval after HBsAg injection when an optimal anti-HBs response was noted, it was necessary to determine the kinetics of the anti-HBs PFU response. In the first experiment (Fig. 1), BALB/c mice were immunized with 40 μg of purified antibodies to the idiotype in saline and then, 7 days later, with 5 μg of HBsAg. Three animals were killed at 4, 10, and 14 days after the HBsAg injection, the spleens were removed, and the number of PFU per spleen was determined. From the data in Fig. 1 it was apparent that the direct anti-HBs PFU response did not differ significantly at the three time intervals examined; however, no indirect plaques were detected on day 4. That the anti-HBs–secreting cells were specific was demonstrated by the fact that no direct or indirect plaques were detected with either uncoated or ovalbumin-coated SRBC at any of the three time intervals. Fourteen days after the mice received their final inoculum was the time interval selected to perform the remaining experiments.

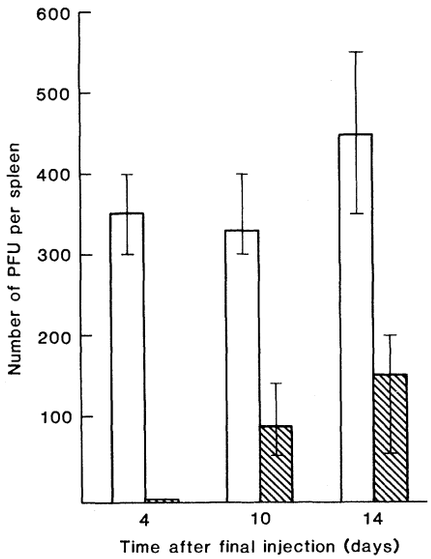

Fig. 1. Kinetics of the anti-HBs plaque-forming units (PFU) response in mice injected with a 40 μg-dose of antibody to idiotype followed 7 days later with an injection of 5 μg of HBsAg. The bars represent the mean PFU (open bar, direct PFU; hatched bar, indirect PFU) per spleen of three mice and the brackets represent the range observed. No direct or indirect plaques were detected with uncoated or ovalbumin-treated SRBC at any time interval.

Five groups of BALB/c mice were treated with antibodies to the idiotype, IgG from nonimmunized rabbits, or HBsAg, and their spleen cells were assayed for PFU response 14 days after the final injection. Injection of antibodies to the idiotype prior to HBsAg exposure generated a significantly higher number of direct PFU (531 as opposed to 183) when compared to mice inoculated with IgG from nonimmunized rabbits (based on a two-tail *t*-test, $P < 0.05$; Table 1). The increase in the mean direct PFU detected in the second group of mice receiving antibodies to the idiotype before HBsAg injection (Table 1) appears to reflect an increase in sample size when compared to the time interval experiment (Fig. 1) (531 from Table 1 as opposed to 450 from Fig. 1). The numbers of indirect PFU obtained in mice that received antibodies to the idiotype or preimmune IgG prior to HBsAg were ten times lower when compared to mice given two injections of HBsAg. However, the group receiving both antibodies to the idiotype and HBsAg did produce a higher number of direct PFU when compared to the group given HBsAg only (531 compared to 167; Kruskal-Wallis, $P < 0.05$). The number of indirect IgG anti-HBs PFU per spleen in the group receiving two HBsAg injections was on the same order of magnitude as those numbers reported for PFU per spleen in secondary responses to HBsAg (*23*) and other viral antigens (*24–26*), thus giving validity to our hemolytic plaque assay as a method for quantitating anti-HBs–secreting cells.

Injection of antibodies to idiotype alone increased by 12 times the number of indirect PFU with anti-HBs specificity when compared to mice that received only preimmune IgG (100 compared to 8.3; Kruskal-Wallis, $P < 0.05$; Table 1). Although the number of direct PFU was higher in the mice that were injected with antibodies to idiotype alone, the number was not significantly different from that obtained with the group treated with

nonimmunized rabbit IgG ($P > 0.1$). The specificity of the PFU response was again demonstrated as no plaques were detected with either uncoated or ovalbumin-coated SRBC in either the direct or indirect assay. The reason for the detection of anti-HBs PFU in only one of six mice that received nonimmunized rabbit IgG for both injections is not known; however, this resulted in the standard error and the mean being equal. The numbers of direct and indirect PFU from the one mouse in this group were low and are probably not meaningful.

Although the standard deviation also exceeded the mean for direct PFU in groups treated with either antibodies to idiotype alone or with nonimmunized rabbit IgG and HBsAg, single-factor analysis of variance by ranks [Kruskal-Wallis test (*27*)] indicated that mean ranks of direct PFU differed significantly ($P < 0.05$) from the group receiving antibodies to idiotype and HBsAg. Such analysis was necessary because of the heterogeneity of the variances within the individual groups. The significance levels obtained by both the parametric two-tail Student's *t*-test and the nonparametric Kruskal-Wallis test were consistent.

We have shown that injection of antibodies to idiotype enhanced the anti-HBs response at the cellular level. An increased number of IgM anti-HBs–secreting cells was obtained by injecting antibodies to the idiotype before antigen exposure. The reason for the increase of cells secreting IgM, but not those secreting IgG, is not known. However, it may reflect the recruitment, by the antibodies to the idiotype, of accessory cells that aid in the induction of a primary anti-HBs response, thus increasing the number of IgM anti-HBs–secreting cells.

It was not surprising that anti-idiotype reagents would alter the anti-HBs activity in immunized animals, as similar results have been obtained in nonviral systems (*3, 8, 10, 12, 13*). Of potential importance was the finding that anti-HBs–secreting cells can be generated by administration of anti-idiotype reagents alone. Such induction of antigen-binding molecules in the absence of exposure to antigen does not usually occur in systems involving soluble antigen after treatment with a heterologous antibody to idiotype (*3, 10, 13*). Recently, however, antibodies to H-2 have been produced in mice by administration of a heterologous antibody to idiotype without alloantigen exposure (*2, 14*).

Anti-idiotype reagents provide an excellent means for modulating the immune response. Studies with these reagents should provide insight into the use of antibodies to idiotypes for manipulation of the immune response so as to provide enhanced protection of hosts from infectious agents. Such success has already been reported with anti-idiotype–induced protection in experimental trypanosomiasis (*11*).

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Nucleotide Sequence and Expression of the Diphtheria *tox*228 Gene in *Escherichia coli*

Abstract. *The complete nucleotide sequence of the diphtheria* tox*228 gene encoding the nontoxic serologically related protein CRM228 has been determined. A comparison of the predicted amino acid sequence with the available amino acid sequences from the wild-type toxin made it possible to deduce essentially the entire nucleotide sequence of the wild-type* tox *gene. The signal peptide of pro-diphtheria toxin and the putative* tox *promoter have been identified, a highly symmetrical nucleotide sequence downstream of the toxin gene has been detected; this region may be the corynebacteriophage* β *attachment site (*att*P). The cloned toxin gene was expressed at a low level in* Escherichia coli.

Diphtheria toxin is secreted as a single polypeptide by *Corynebacterium diphtheriae*, lysogenic for bacteriophage β^{tox+}, and is subsequently cleaved into two fragments, A and B (*1*). Fragment A blocks protein synthesis in most eukaryotic cells by specifically catalyzing an adenosine diphosphate (ADP)-ribosylation of elongation factor 2. Fragment B interacts with a membrane receptor for toxin and is essential for the delivery of fragment A into the cytoplasm. The toxin gene has previously been localized on the β-phage genome (*2, 3*), and the amino acid sequence of fragment A has been determined (*4*). Various mutant β phages have been isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) mutagenesis, and these encode nontoxic cross-reacting materials (CRM). Corynebacteriophage $\beta^{tox-228}$ carries the *tox*228 allele and encodes CRM228, which is serologically indistinguishable from the wild-type toxin (*5*). However, CRM228 is devoid of enzymatic activity and has a reduced receptor binding capacity.

We have cloned in *Escherichia coli* a 3.9-kb Bam HI fragment of DNA from the clear plaque-forming $\beta_c^{tox-228}$ in plasmid pBR322. We have localized the toxin gene on this fragment and have determined the complete nucleotide sequence of the *tox*228 allele and adjacent regions. The DNA sequence (Fig. 1) reveals a single open reading frame that is capable of encoding a protein with a molecular weight of about 61,000, corresponding to diphtheria toxin. The amino acid se-